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THE MECHANISM OF PINOCYTOSIS IN THE RAT VISCERAL YOLK SAC

by

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fulfilment of the requirement for Degree of Doctor of Philosophy.

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The following was redacted from this digital copy of the original thesis at the request of the awarding university:

Appendix III

Appendix IV

Appendix V

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ABSTRACT.

The rat visceral yolk sac cultured in vitro was used as model system to study the mechanism of pinocytosis. Fluid-phase pinocytosis was completely inhibited at low temperature and on addition of metabolic inhibitors to the culture medium, was partially inhibited by cytochalasin B and colchicine and was also reduced to various extents by other factors such as EGTA and theophylline (Chapter 4). Polycations have been reported in the literature as stimulators of endocytosis. Here it was found that poly-L-ornithine, poly-L-lysine and DEAE-dextran stimulated tissue-association of colloidal ^{198}Au (Chapter 5) by a mechanism which involved the formation of a polycation-colloidal ^{198}Au complex that bound to the outside of the visceral yolk sac (Chapter 6). Electron microscopical visualization of tissue which had been exposed to polycation-colloidal Au complexes did not reveal colloidal Au in association with the plasma membrane and potential explanations of this observation are discussed in (Chapter 7). DIVEMA is a synthetic polymer which has a wide range of biological activities although its mechanism of action is still obscure. Three different molecular weight distributions of DIVEMA and three DIVEMA derivatives had no stimulatory effect on pinocytosis in the rat visceral yolk sac; in some instances they were inhibitory (Chapter 8). None of the above substances were found to stimulate the rate of pinosome formation in the visceral yolk sac.

A method for radiolabelling PVP was evaluated (Chapter 9) and it was found possible to iodinate PVP with acceptable efficiency. Excess radioiodide was removed by dialysis, but the resultant preparations of ^{125}I -PVP showed spontaneous deiodination when stored at 4°C . The increase in radioiodide in preparations during a 6.5h culture period at 37°C was fairly small, so a technique was developed for quantitation of pinocytosis of these ^{125}I -PVP preparations. Four samples of PVP with different molecular weight distributions were iodinated and preliminary experiments were carried out to investigate their rates of pinocytosis (Chapter 10). A correlation was observed between increasing molecular weight and a decreased rate of pinocytic uptake of PVP.

The study of the effects of polyamino acids, dextran derivatives and DIVEMAs on pinocytosis in the rat visceral yolk sac was paralleled by a study of the effect of these compounds in pinocytosis in the rat peritoneal macrophage (cultured in vitro) which was carried out by Dr. M.K. Pratten in this laboratory. The work of Dr. Pratten is referred to extensively in this thesis as the results described for the visceral yolk sac system are of wider interest when viewed comparatively.

Some of the experimental data and theoretical concepts reported in this thesis have been published as follows:-

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³Duncan, R., Pratten, M.K. & Lloyd, J.B. (1979) Mechanism of Polycation Stimulation of Pinocytosis, Biochim. Biophys. Acta in press.

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1 Appendix III

2 Appendix IV

3 Appendix V

ABBREVIATIONS.

| | |
|--------|--|
| ATP | Adenosine triphosphate |
| BSA | Bovine serum albumin |
| dBSA | Formaldehyde denatured bovine serum albumin |
| cAMP | Cyclic adenosine monophosphate |
| DIVEMA | Divinyl ^{ether} maleic anhydride |
| DMF | Dimethyl formamide |
| EGTA | Ethylene glycol-bis (β -amino ethyl ether)- N,N' - tetra acetic acid |
| EI | Endocytic Index |
| HSA | Human serum albumin |
| PVP | Polyvinylpyrrolidone |
| TCA | Trichloroacetic acid |
| HDL | High density lipoprotein |
| LDL | Low density lipoprotein |

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CHAPTER 1

GENERAL INTRODUCTION.

1.1. ENDOCYTOSIS

Membranes give cells their individuality by separating them from their environment, and this compartmentation has been necessary for the functional specialization achieved during the course of evolution. The presence of a discreet boundary between the cell and its environment necessitates the existence of a wide variety of transport mechanisms to enable movement of substances from one side of the boundary to the other. Whereas small molecules gain entry readily by either passive diffusion or an active transport system, macromolecules and particles cannot pass via this route. To overcome this problem the cell has developed a transport mechanism that involves the movement of the plasma membrane in such a way that membrane-bounded vesicles pinch off into the cytoplasm capturing extracellular fluid, particulate matter, or both. This mechanism of transport is now known as endocytosis (de Duve, 1963).

Many different terms have been employed to describe different types of endocytosis and these are listed extensively by Chapman-Andresen (1962) and Jacques (1969), but in general they fall into two broad categories, phagocytosis and pinocytosis. The term phagocytosis was first coined by Metchnikoff (1883) to describe the ingestion of particles by leucocytes and is associated with the uptake of particulate matter such as bacteria, latex beads and erythrocytes by specialized metazoan cells such as macrophages and also unicellular organisms. These are all processes visible with a light microscope. Meltzer (1904) first envisaged the possibility of endocytosis at the sub-light microscope level in a hypothesis suggesting fluid uptake and passage through cells as an explanation of oedema in mammalian tissues, but it was Warren Lewis (1931),

following his observation of mammalian cells cultured in vitro who first proposed the mechanism and called it pinocytosis.

Pinocytosis is a process by which the cell engulfs small droplets of extracellular fluid and its contents, and was first demonstrated in blood capillaries by Palade (1953) using electron microscopy. Pinocytosis is now known to occur in many cell types including amoebae, macrophages, fibroblasts and many epithelia including intestinal and kidney tubule cells.

Although there are classical differences between the two types of endocytosis and it is easy to differentiate between an amoeba engulfing its prey and a cell internalizing microscopic droplets of fluid, it is difficult in many instances to categorise an endocytic event. In general it is now agreed that the differences between phagocytosis and pinocytosis are probably superficial, simply being a reflection of the vesicle size involved, with the same underlying cellular mechanism responsible in both cases. All types of endocytosis show a common sequence of events and these are as follows:-

1. Membrane Internalization: In response to an internal or external trigger (such as particle attachment), the plasma membrane invaginates or spreads outwards to engulf the substrate. The infolding edges of plasma membrane fuse and the newly formed vesicle is then released in the cytoplasm.
2. Translocation: Vesicles forming at the plasma membrane migrate towards the perinuclear region. At this time the incoming vesicles may fuse with each other and subsequently fuse with primary lysosomes to form secondary lysosomes. These can then

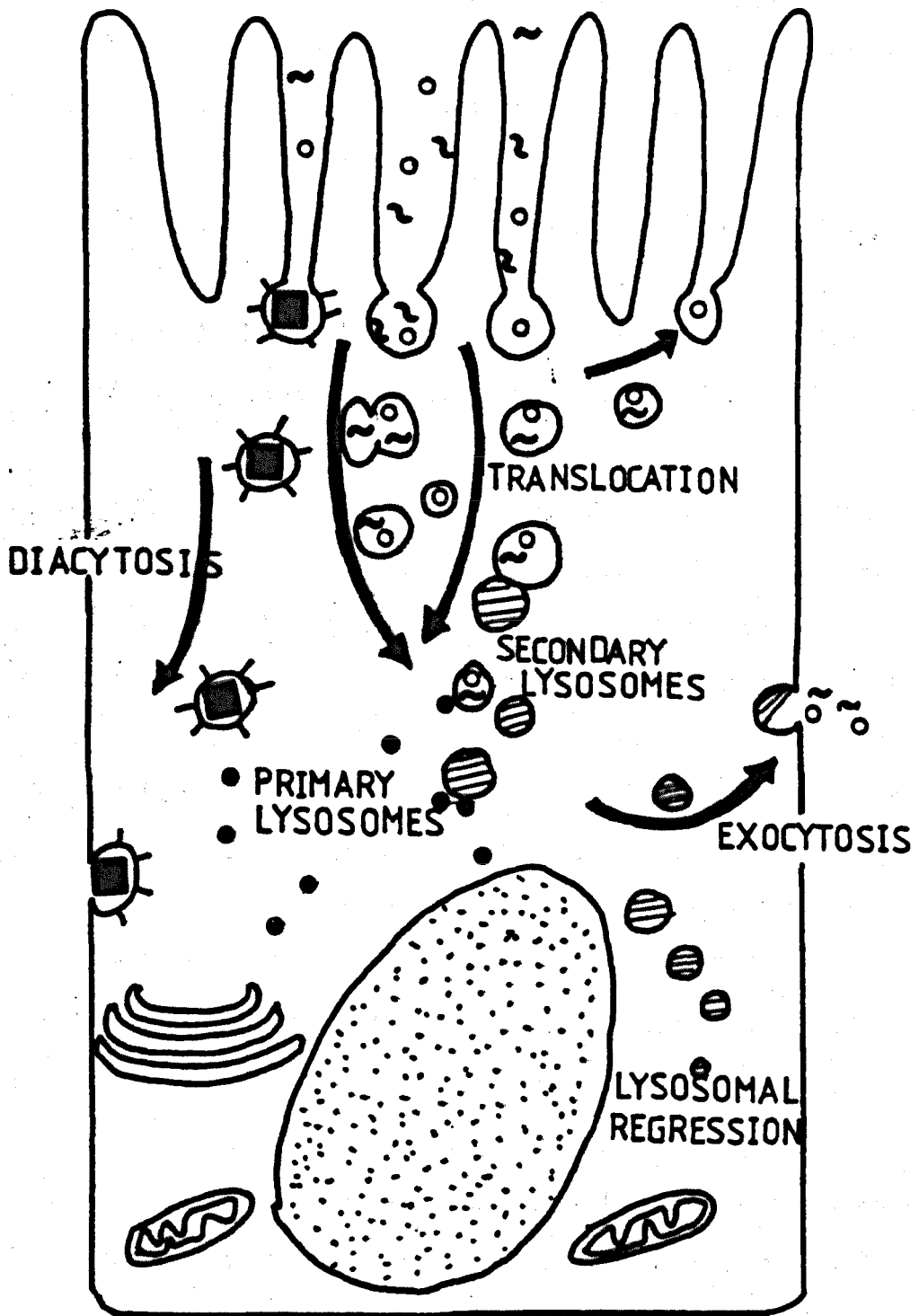
enter into the general fusion sequence. Exposure of vesicle contents to lysosomal hydrolases results in the catabolism of degradable material.

3. Lysosomal Regression: Small molecules released by degradation escape through the lysosomal membrane, whereas large non-degradable material is accumulated to form residual bodies, unless the cell can regurgitate this material using an exocytic mechanism.

Fig. 1.1. shows a simplified diagram of a typical endocytic cell.

In this thesis the rat visceral yolk sac was used as a model to study endocytosis. The columnar epithelial cells that are active in endocytosis have apical microvilli which probably restrict the tissue to the formation of relatively small vesicles at the plasma membrane i.e. pinocytosis. The rest of this section (1.1.1. - 1.1.3.) describes the endocytic pathway in detail with particular reference, where possible, to pinocytosis. Much of the evidence which provides an insight into the relevance of certain stages in the interiorization process has been derived from phagocytic systems and as yet comparative studies with pinocytic systems have not been undertaken.

FIG.1.1. A TYPICAL ENDOCYTIC CELL



○ Substrate with no membrane affinity

■/~ Substrate with membrane affinity

1.1.1.1. Membrane Internalization

In many endocytic processes, particularly phagocytosis, substrate attachment is a prerequisite for membrane internalization. Observation of phagocytically active cells with the light microscope and both scanning and transmission electron microscopes have shown the plasma membrane in very close association with ingested particles, and more recent studies confirm the existence of a distinct attachment step separable from ingestion. Rabinovitch (1967) showed that attachment and ingestion of glutaraldehyde-fixed erythrocytes by macrophages were distinguishable on the basis of their metabolic characteristics, requirement for serum factors, and also divalent cations.

Before attachment of any substrate to the membrane there can be some degree of discrimination and the mechanism of recognition involves the concept of membrane receptors. A receptor is an area within the membrane, composed of a single component or several components, which presents a defined structure at the membrane surface that can bind to a specific moiety on an extracellular particle or molecule. If these receptors can only bind to a very limited range of molecules or a single molecule they are termed "specific" receptors, or conversely if they bind a wide range of compounds they are designated "non-specific" binding sites. The chemical nature of receptors varies. Allen & Cook (1970) showed that the receptors for opsonized bacteria in macrophages are proteins and Lay & Nussenzweig (1968) demonstrated three separate receptors on the macrophage membrane by their varying sensitivity to trypsinization and local ionic strength. These receptors appear to show specificity towards complement, IgM and IgG.

In general three main factors affect the attachment phase. Firstly the surface charge on the particles can either facilitate or hinder uptake. Amoebae (Chapman-Andresen, 1977) carry a net negative charge on their membrane surface, so positively charged particles are attracted. Secondly the presence of hydrophobic groups on the surface of the particle can increase its affinity with the plasma membrane (Rabinovitch, 1970) and finally physical size of the ingested particle also plays a role in the attachment phase. Korn & Weismann (1967) have studied the uptake of latex beads by Acanthamoeba and they found that a specific amount of latex must bind to the surface before phagocytosis could proceed, the optimum size for phagocytic vesicles being approximately the same for beads 0.088 - 2.68 μm in diameter. In a pinocytic system there will be a relatively low upper size limit to the particle size that can enter the small newly formed vesicles. The existence of a specific receptor for a substance (involving one or more of the aforementioned factors) greatly enhances the chance of binding. This is of great importance in phagocytosis by macrophages and polymorphonuclear leucocytes which have receptors for certain antibodies and complement components which enable them to carry out what has been termed "immunological" phagocytosis (Rabinovitch, 1970).

It seems clear that the attachment of particles to the plasma membrane initiates internalization, at least for phagocytosis, but the actual transduction mechanism which relates the binding event to the actual membrane movement remains unclear. Christiansen & Marshall (1965) showed that prolonged contact of Chaos chaos with

its prey was required to stimulate food cup formation. An excitation-contraction coupling mechanism as described for muscle may also be of importance in endocytosis. Resting potentials across the membrane of endocytic cells have been reported at -13mV, -20mV and -80mV (Gallin et al., 1977; Allison & Davies, 1974; Josefsson et al., 1975) and it is suggested that there is a correlation between membrane polarization and the intensity of pinocytosis. Josefsson et al., (1975) found that pinocytic channels began to appear in Amoeba proteus when the membrane was depolarized to -30mV and with all inducers the maximum pinocytosis was observed at membrane potentials close to zero. Allison & Davies (1974) proposed a mechanism by which particles binding to glycoproteins in the membrane caused immobilization of these units and thus an ion flux could pass across the membrane.

Owing to their role in muscle, calcium and cAMP have been examined as possible candidates for the secondary messengers in the endocytic system. However, as pointed out by Stossel (1977), these are soluble compounds and they might be expected to produce a diffuse response throughout the cell unless there were some very stringent local control mechanisms.

In the case of phagocytosis much evidence suggests that ingestion is a localized response. Macrophages ingesting latex and antibody coated bacteria will not take up non-specifically bound erythrocytes at the same time (Griffin & Silverstein, 1974) and similarly erythrocytes only half coated with IgG are only partially interiorized (Griffin et al., 1975). Dibutryl cAMP and other compounds which increase the intracellular cAMP level,

generally have an inhibiting effect on endocytosis. Cox & Karnovsky (1973) showed that theophylline, dibutyl cAMP and prostaglandins inhibited the phagocytosis of starch particles by guinea pig polymorphonuclear leucocytes and similar results were obtained in a pinocytic system by Brown & Segal (1978) who showed that the uptake of invertase was sensitive to inhibition by glucagon and epinephrine. An exception to the general trend is the uptake of horseradish peroxidase by isolated toad urinary bladder which is stimulated by oxytocin, dibutyl cAMP and theophylline (Masur et al., 1971). Cyclic nucleotides may mediate endocytosis in certain cell types, perhaps those which rely on cAMP to control more general aspects of their metabolism. Recent studies have actually measured intracellular levels of cAMP during phagocytosis but again the evidence is contradictory. Although Seybeth et al., (1973) found that the cAMP content of pig peripheral leucocytes did not change on incubation with latex beads, alveolar macrophages exhibited a significant increase as early as 30s after the addition of particles. Manganello et al., (1971) found an increase in cAMP concentration in crude leucocyte preparations during phagocytosis of the same substrate.

The effect of divalent cations on pinocytosis is also dependent on cell type and substrate used. Human leucocytes and alveolar macrophages require divalent cations in the extracellular medium to digest unopsonized or opsonized albumin particles (Stossel, 1973). In other systems calcium plays a more important role in membrane binding than ingestion. Complexes formed between labelled proteolytic enzymes and α -macroglobulins require divalent cations to facilitate binding to the membrane (Debanne et al., 1976) and

calcium is a prerequisite for transferrin association with reticulocytes (Hemmaplardh & Morgan, 1977).

Much of the data relating to the transduction mechanism of endocytosis is contradictory, different cell types displaying contrasting responses. The role of cyclic nucleotides and divalent cations is still unclear, but even if they do have a definitive function, the way that they achieve membrane propulsion has yet to be unravelled. Most workers in this field are basing their investigations on the assumption that a skeletal muscle model may be basically applicable to the endocytic mechanism, and this may not be the case. It is noteworthy that many of the factors under investigation have already been shown to have a role in the secretory mechanism which in many ways is closely related to endocytosis.

Although attachment of particles to the plasma membrane initiates internalization in phagocytic cells, time-lapse cinematography of many pinocytic cell types indicate a more or less constant movement of the cell membrane, so perhaps some types of pinocytosis are either ongoing processes or they rely on some less obvious trigger. Ingestion follows the attachment and transduction stages, at which time there is a movement of the plasma membrane which results in the pinching off of a section of membrane to form a vesicle or vacuole. This movement can take one of two forms, either the formation of pseudopodia which gradually spread out and entrap an object, commonplace in phagocytosis, or alternatively there can be an invagination which fuses at the cell periphery usually seen in pinocytosis. It is interesting to consider whether the membrane moves due to intrinsic contractile properties or if it is propelled by some external force. Two types of movement can occur in membranes themselves (Allison &

Davies, 1974). Alteration of ionic composition of the medium leads to a wavy motion in the lamellae of liposomes (Bangham et al., 1965), but this is unlikely to be of importance here. It is now generally accepted that the lipid and protein components of membranes are able to move laterally within the confines of the membrane at temperatures above the transition temperature of the membrane and this could well be of relevance to endocytosis (Singer & Nicholson, 1972)

It has been shown that there is lateral diffusion of receptors within the plane of the membrane to form "patches" and "caps" in lymphocytes and such grouping of receptors could lead to distortion of the membrane. Probably a large part of membrane movement is achieved by extrinsic forces. Goldman & Folett (1969) first suggested that the bundles of microfilaments, seen beneath the plasma membrane using electron microscopy, are the basis of a contractile system which facilitates membrane movement, and that microtubules are the cytoskeletal support system to which the filaments are attached and which they contract against. This idea seemed more likely when it was shown that the fungal metabolite cytochalasin B, which is believed to inhibit microfilament systems, also inhibited phagocytosis by mouse peritoneal macrophages (Allison et al., 1971) and polymorphonuclear leucocytes (Klaus, 1973). An alternative method of membrane propulsion could be the direct effect of binding multivalent ligands onto the plasma membrane. Recently Hewitt (1977) suggested a theoretical model for the induction of membrane curvature by the binding of polyvalent ligands to sites on the membrane surface. If this were the only way of inducing the membrane movements necessary to produce a vesicle, the number of substances endocytosed would be very limited, in fact strictly limited to those multivalent compounds which would bind the plasma membrane in this way and any soluble compounds which accompanied their entry.

1.1.2. Translocation

Once formed, the endocytic vesicles migrate towards the centre of the cell. Green & Casley-Smith (1972) calculated that small vesicles traversing endothelial cells could achieve sufficient energy for their movement through their own Brownian motion and they suggested that the small vesicles pass slowly through cells in a completely random fashion. Others have postulated that vesicles move towards the perinuclear region in a more orderly fashion, possibly streaming along the microtubule network. However migration is achieved, it is known that the incoming pinocytic vesicles frequently fuse with each other (Cohn et al., 1966). It is quite likely that some vesicles also fuse again with the plasma membrane returning their contents to the extracellular space. This phenomenon would be difficult to detect from micrographs at either the light or electron microscope level or even assess quantitatively using any other technique.

The fate of vesicles within the cell is usually to enter the lysosomal system, this again being achieved by the process of fusion. Incoming pinosomes fuse with either small primary lysosomes or the larger secondary lysosomes. Willingham & Yamada (1978) recently carried out a study of cultured mouse fibroblasts using the phase-contrast microscope. They commented on the fact that the intracellular organelles moved with great speed and that not only did lysosomes collide with and fuse with pinosomes but they also appeared to cause them to fragment into smaller pieces. This process was called "piranhalysis".

It was Metchnikoff over a hundred years ago who first noticed that phagocytosed compounds reached digestive vacuoles within the cell.

De Duve (1955, 1959) first developed the concept of lysosomes, suggesting that the enzymes they contain were catabolic in nature and that the lysosomal membrane simply serves to segregate these enzymes from the cell to prevent damage. Since it has been shown that lysosomes contain a large number of hydrolytic enzymes and these have been recently reviewed by Barrett & Heath (1977). Some enzymes are located in the interior matrix of the lysosome and some bound to the inner surface of the lysosomal membrane. All the major categories of naturally occurring complex molecules entering the cell in pinosomes would seem to be substrates for lysosomal enzymes.

Substances entering by endocytosis which are not degradable accumulate within the lysosomal compartment of the cell. In vivo this phenomenon is seen in certain disorders known as lysosomal storage diseases which are mainly due to a deficiency in a certain degradative enzyme, and result in the accumulation of abnormal amounts of macromolecules within intracellular bodies and this is often accompanied by the presence of high levels of similar materials in the blood circulation. In vitro models of lysosomal storage diseases have been produced by administering indigestible lysosomotropic agents such as dextran, Triton WR-1339 and sucrose (Lloyd 1973; Roberts et al., 1976).

In certain specialized systems the incoming pinosomes appear to be programmed to traverse the cell, carrying material from one side to the other and this mechanism has been termed diacytosis. The process has been visualized in endothelial cells facilitating the passage of ferritin (Bruns & Palade, 1968) and has been proposed

as a method for the transport of immunoglobulin via the rodent yolk sac (Wild, 1975). It is not known whether such pinosomes interact with lysosomes before re-fusing with the cell membrane, but it is likely that at least a few may escape this fate if movement is rapid and fusion random.

1.1.3. Lysosomal Regression.

The eventual fate of the lysosome could be one of two possibilities. It could disappear from the cell by either exocytosis or some involution mechanism, or alternatively it could persist within the cell. Probably each of these mechanisms occur. Acanthamoeba which has phagocytosed latex beads has been shown to exocytose them during the encystment stage of its life cycle (Stewart & Weisman, 1972). Regression may occur by loss of fluid from the secondary lysosome and this may be accompanied by budding inwards of the lysosomal membrane, with the consequence that membrane be degraded to its monomeric components (Hayashi et al., 1973). Exocytosis via the lysosomal compartment would result in the loss of non-membrane-bound lysosomal hydrolyases and the incorporation of lysosomal membrane into the plasma membrane could cause intermixing of potentially distinct membrane components. It is possible that this "lysosomal membrane" could be rapidly recycled by selective interiorization in the form of a pinosome. Lysosomes may also be lost from the cell by a mechanism of ejection with the elimination of intact lysosomes and it has been suggested that the tumoricidal effect of activated macrophages may be mediated by transference of intact lysosomes from the macrophages into the target cell (Hibbs, 1976).

1.2. QUANTITATION OF ENDOCYTOSIS.

Although much of the work on endocytosis has been descriptive in nature, over the past fifty years many workers have tried to find suitable techniques by which the endocytic capture of substances might be quantitated. Until recently the methodology employed was generally somewhat over simplified and the interpretations made open to criticism. Three main types of cell have been used to study endocytosis. The large unicellular Protozoans such as Amoeba proteus, Chaos chaos and Tetrahymena pyriformis have been investigated extensively (Chapman-Andresen, 1971; Bowers & Olszewski, 1972; Ricketts, 1971). These cells are particularly interesting, as their normal feeding cycle involves the capture of food particles by a phagocytic mechanism, but they are also able to pinocytose. As they are so large, it is relatively easy to visualize the endocytic event and this is one advantage of the system. One potential disadvantage could be the discontinuous nature of induced pinocytosis, which only lasts for about thirty minutes in Amoeba proteus (Chapman-Andresen, 1971). A second group of cells that have been widely studied is those that fall into the category of mononuclear phagocytes. These cells have a variety of names depending on their situation within the mammal and they are widely distributed throughout the body, being found in the blood, liver, lungs, spleen, and peritoneal cavity. Cells with a variety of origins have been investigated: Kupffer cells (liver, Munthe-Kaas, 1977), peritoneal macrophages (Cohn, 1966) and alveolar macrophages (Gormley et al., 1978) to name but a few. More recently work has begun which investigates pinocytosis by mammalian epithelia cells such as those found in the duodenum, kidney tubule and yolk sac. Owing to their fixed position and microvillous apical plasma membrane these cells are more limited as to the size of

particles they can engulf and they are probably restricted to pinocytosis. It was not until the advent of electron microscopy that pinocytic vesicles could be seen within these cells and this explains why quantitation in these systems is a more recent innovation.

Endocytic uptake has been quantitated using both in vivo and in vitro systems. Most in vivo work involves the intravenous injection of particulate matter and a subsequent measurement of clearance rate. Post mortem examination reveals the concentration of particles largely by the liver and spleen. These procedures are not discussed in detail, as the work reported in this thesis is concerned with an in vitro system. By definition an in vitro system involves the use of cell, tissue, or organ culture technique to maintain living cells in a near normal physiological condition whilst in isolation from the parent animal. Some methods use a cell suspension technique whilst others require the adhesion of cells to some type of support platform. Once a stable culture system has been set up the endocytic properties of the cells can be examined.

Two main approaches have been used to quantitate endocytosis in vitro:

1. Morphometric methods, using light or electron microscopy when the following can be estimated and used to infer endocytic rates of uptake:

- (i) the number of endocytic vacuoles within a cell.
- (ii) the number of particles within a cell.
- (iii) the number of cells within a population that have taken up particles.

Although there are advantages in being able to observe cells, the interpretation of morphometric data is not always simple. When looking at cells in one plane it is difficult to assess whether circular profiles are in fact vesicles or alternatively if they are transverse sections of canaliculi which are in communication with the extracellular space. Only serial sections would confirm one of those alternatives. Having decided what are in fact vesicles within the system, extrapolation from vesicle numbers within a cell to endocytic rate again has its pitfalls. The number of vesicles present within a system at any one time depends not only on the rate of formation of those vesicles but also on their rate of disappearance and any change in the longevity of vesicles could certainly change vesicle number. Another important determinant of the number of vesicles visible within a cell is the resolution of the microscope used. Cohn and co-workers (1966, 1967 a,b,c.) carried out a large number of studies on pinocytosis by the mouse peritoneal macrophage using vesicle number as measured by phase-contrast microscopy as an indication of pinocytic rate. Recently, it has been stated that only 16% of the incoming vesicles within mouse peritoneal macrophages are detectable with the phase-contrast microscope (Steinman et al., 1976). As endocytic vesicles are thought to increase in size by the process of fusion, any factor which inhibited vesicle fusion but did not affect the rate of vesicle formation would obviously produce misleading data if a morphometric method were being used to estimate pinocytic rate.

2. Direct measurement of substrate capture, using a marker which is either radiolabelled or detectable chemically or enzymically. Various markers have been used for this purpose in a variety of

systems. Some examples are summarized in Table 1.1.

The use of a radiolabelled substance or a chemical marker is usually a more efficient method of quantitation, but these techniques are not without problems. One must be sure that the marker used is in fact penetrating the cell by endocytosis and not just progressively binding to the exterior of the cell or lodging within intercellular spaces. In some cases this question can easily be answered by simultaneous microscopical examination of the system. Certain criteria have been suggested to confirm the existence of endocytosis and these include a constant rate of progressive accumulation of substrate over a long time period (binding would be rapidly saturable), inhibition of uptake at low temperature and in the presence of metabolic inhibitors (binding would not require metabolic energy) and a low rate of release of substrate from the system when a pre-incubation phase in the presence of substrate is followed by incubation in the absence of substrate (binding would have a substantial rapidly reversible component).

Degradable macromolecules that penetrate the cell via endocytosis are liable to subsequent hydrolysis within the lysosomal system. Consequently to calculate the pinocytic uptake of such substances one must measure both the tissue level of the substance, and the release of digestion products back into the culture medium. The sum of these two factors then gives the true pinocytic rate of a degradable substance. Studies by Ryser & Hancock (1965) investigated the uptake of ^{131}I -labelled human serum albumin (^{131}I -HSA) by sarcoma-180 cells but here they failed to take the catabolic effect into account so the data obtained did not really relate to the net

TABLE 1.1. SYSTEMS USED TO QUANTITATE ENDOCYTOSIS DIRECTLYPHAGOCYTOSIS

| Marker | Endocytic System | Reference |
|---|-----------------------------------|-------------------------------------|
| Polystyrene latex beads | Polymorphonuclear leucocytes | Roberts & Quastel (1963) |
| Polystyrene and Polyvinyl toluene latex beads (dioxane extraction) | Acanthamoeba | Weismann & Korn (1967) |
| [¹⁴ C] Acetyl or [¹⁴ C] Methyl Starch Particles ³² P <u>Salmonella typhimurium</u> | Polymorphonuclear leucocytes " | Michell <u>et al.</u> , (1969) " |
| Paraffin oil coloured with Oil Red O (dioxane extraction) | Alveolar Macrophages | Mason <u>et al.</u> , (1973) |
| Paraffin oil droplets containing Oil Red O and coated with <u>Escherichia</u> <u>coli</u> lipoprotein | Human leucocytes | Stossel (1973) |
| ⁵¹ Cr-labelled Erythrocytes | Mouse peritoneal macrophages | Manovani <u>et al.</u> , (1972) |
| Immune (¹²⁵ I-labelled) Complexes | Rabbit leucocytes | Ward & Zvaifler (1973) |
| Staphylococcal protein A- IgG(¹²⁵ I-labelled) | Human neutrophils | Hällgren & Stalenheim (1976) |

contd

TABLE 1.1. contd.

PINOCYTOSIS

| Marker | Endocytic System | Reference |
|---|---------------------------------|---------------------------------------|
| <u>Radiolabelled macromolecules:-</u> | | |
| ^{125}I -PVP | Aortic Smooth Muscle | Leake & Bowyer (1977) |
| ^{125}I -LDL | Normal Human Fibroblasts | Miller <u>et al.</u> , (1977) |
| ^{125}I -HDL | | |
| ^{131}I -Human serum albumin | <u>Acanthamoeba castellanii</u> | Bowers & Olszewski (1972) |
| <u>Others:</u> | | |
| Ferritin | Sarcoma 180 cells | Petitpierre-Gabathuler & Ryser (1975) |
| Invertase | Rat visceral yolk sac | Brown & Segal (1978) |
| Horseradish Peroxidase | Fibroblasts (three cell lines) | Steinmann <u>et al.</u> , (1974) |
| α -Macroglobulin-Protease Complexes (quantitation by rocket immunoelectrophoresis) | Human Fibroblasts | Leuven <u>et al.</u> , (1978) |

pinocytic rate of the substrate used. Later, more sophisticated studies do take account of this phenomenon (Williams et al., 1975 b; Miller et al., 1977).

In conclusion there are probably two main factors to consider when trying to establish a reliable system for quantitation of endocytosis in any cell type. Firstly it is important to determine the specificity of a substrate as a pinocytic marker and secondly the intracellular stability of the substrate must be measured. A degradable marker can be suitable, but allowances must be made for any loss of activity due to hydrolysis. The most reliable methods probably utilize a biochemical approach, but simultaneous histological examination of the system is certainly advantageous. Markers can be chosen, which are detectable both biochemically and histologically, so that the intracellular location of substrate can be checked.

1.3. THE RAT YOLK SAC

1.3.1. Morphology

Two types of placenta exist concurrently in the rat, the chorioallantoic placenta and the inverted yolk sac placenta, and both of these structures begin to differentiate from the blastocyst at approximately six days of gestation, the chorioallantoic placenta becoming more prominent after day eleven. Fig. 1.2. shows the progressive development of the embryonic membranes of the rat during gestation. The inverted yolk sac placenta consists of two distinct structures, the outer parietal yolk sac and the inner visceral yolk, separated by the yolk sac cavity. Before the sixteenth day the parietal yolk sac disappears leaving the visceral yolk sac in communication with the uterine cavity.

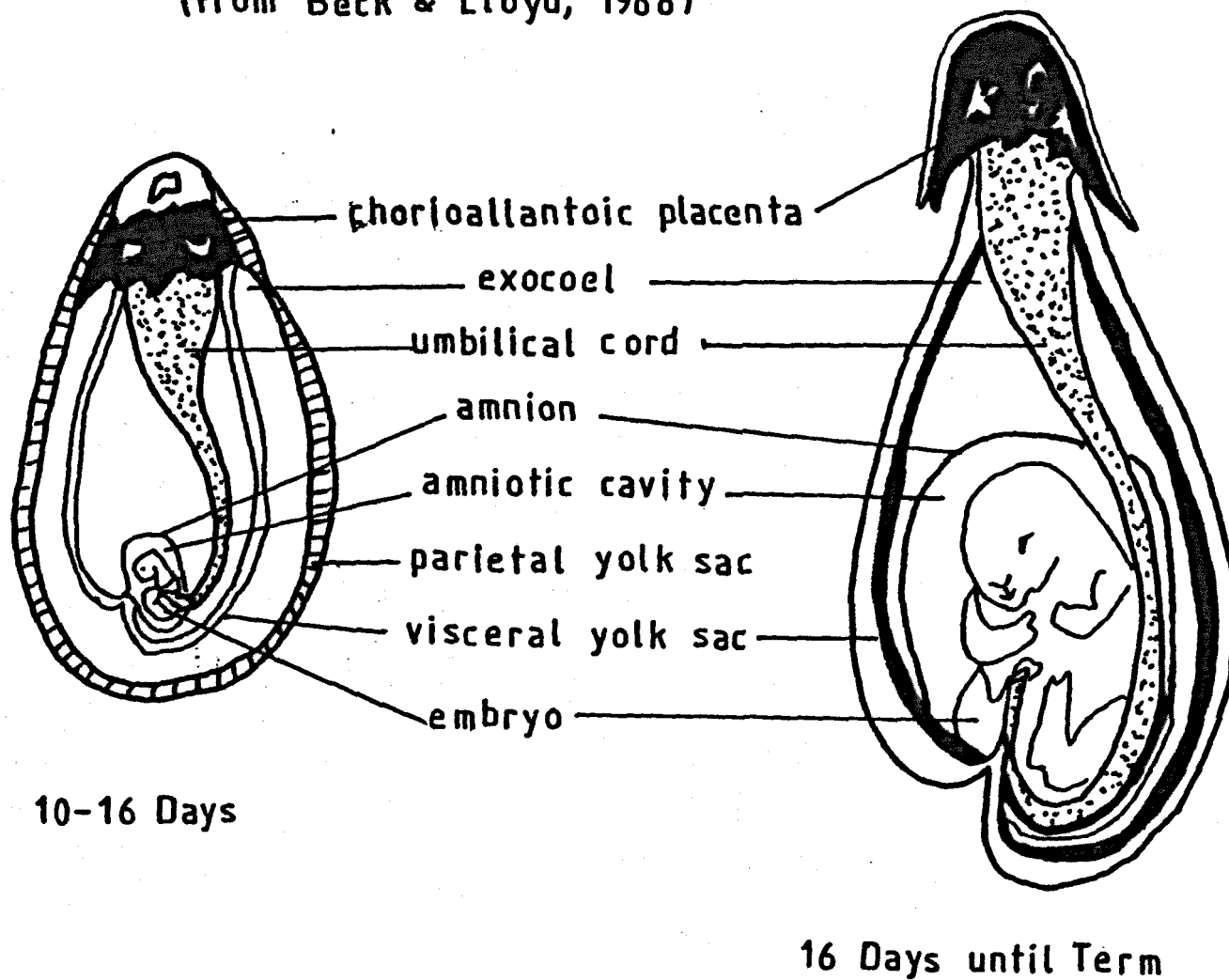
The visceral yolk sac is composed of three separate cellular layers as follows:-

i. Outer visceral endoderm composed of columnar epithelial cells which rest on a basement membrane. At day eleven the surface of the endoderm has only a few short microvilli, but by the sixteenth day the cells have become much taller and the microvilli have increased in number and size. This trend towards increased folding of the apical membrane is continued until term.

ii. The second layer is called the mesenchymal layer. Its composition depends on the stage of development and the area of the wall under consideration, but generally it consists of scattered mesenchymal cells interspersed with vitelline blood capillaries. Towards term the number of fetal capillaries increases. This layer rests on a thick fibrous sheet called the serosal basement membrane.

FIG. 1.2. THE DEVELOPMENT OF FETAL MEMBRANES IN THE RAT

(from Beck & Lloyd, 1968)



iii. Internal to the serosal membrane are a few scattered flattened mesothelial cells which line the exocoelom.

Fig. 1.3. shows the relationship between the cellular layers of the visceral yolk sac at approximately 17.5 days.

It is the columnar epithelia and cells of the visceral endoderm which are active in pinocytosis, and an electron micrograph and a diagrammatic representation of one such cell at 17.5 days are shown in Figs. 1.4 and 1.5. Directly beneath the highly microvillous apical border is a complex canalicular system composed of invaginations of the plasma membrane which form caveolae that sometimes form complex interconnections. These infoldings are often coated with a filamentous layer (Jollie & Triche, 1971) and it has been postulated that this is a polysaccharide coating responsible for selectivity in pinocytosis of different compounds (Bennet, 1956). Many small pinocytic vesicles, less than $0.2\ \mu\text{m}$ in diameter) are visible in this region of the cell (Lambson, 1966); some are electron dense whilst others are electron lucent and also have a "fuzzy coating" which it has been proposed is arranged into a polygonal network (Ockleford, 1976). The latter have been named coated vesicles and are probably derived from the caveolae. Towards the perinuclear region the vesicles become considerably larger in diameter ($1 - 2\ \mu\text{m}$) usually containing electron-dense material, and sometimes containing myelin figures (autophagic vacuoles).

The nuclei of these cells are commonly basally located and Golgi elements occupy a paranuclear position, primarily laterally and basally disposed. Lipid droplets are often present between

FIG. 1.3. THE RAT VISCERAL YOLK SAC (17.5 day) AS SEEN WITH THE
LIGHT MICROSCOPE

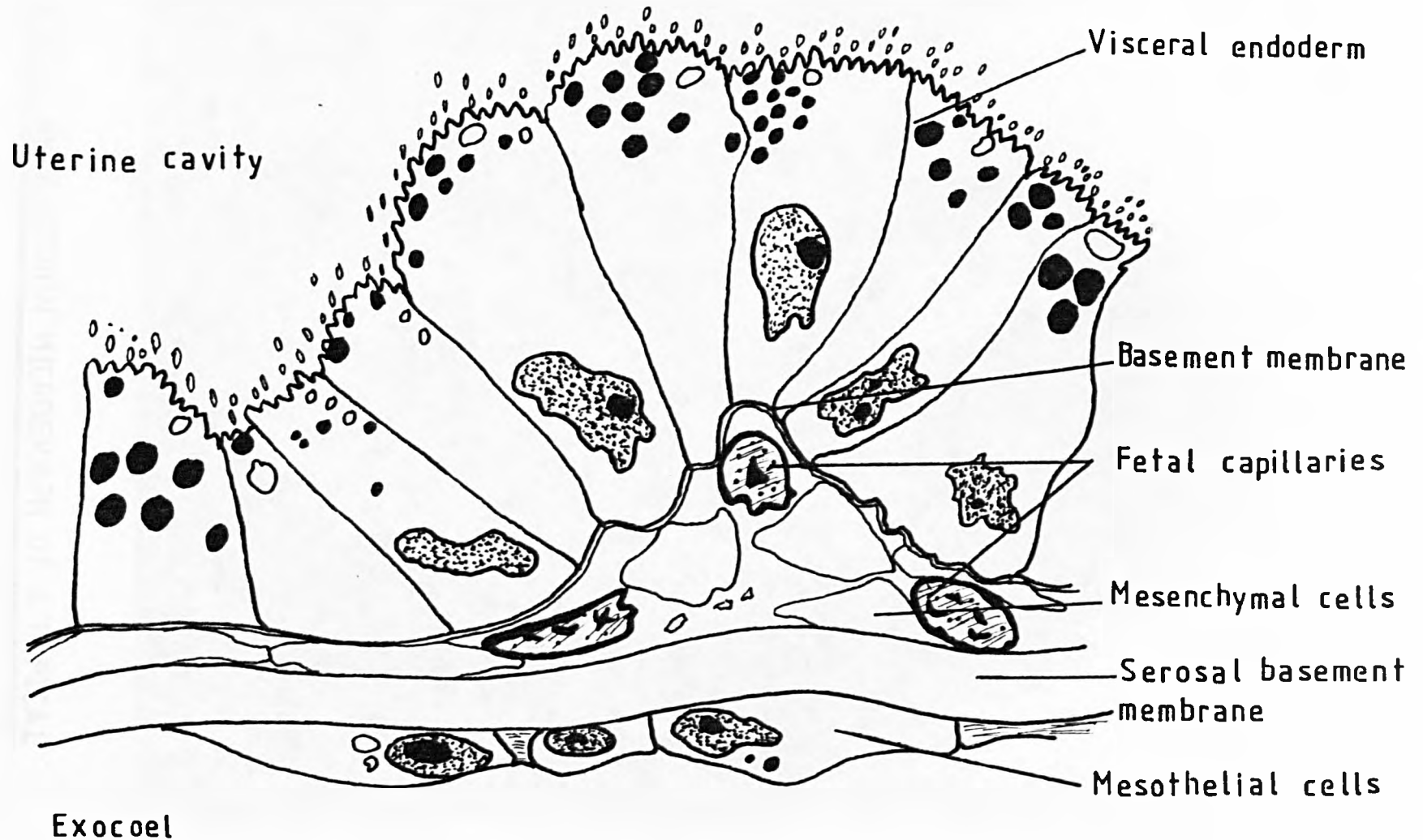
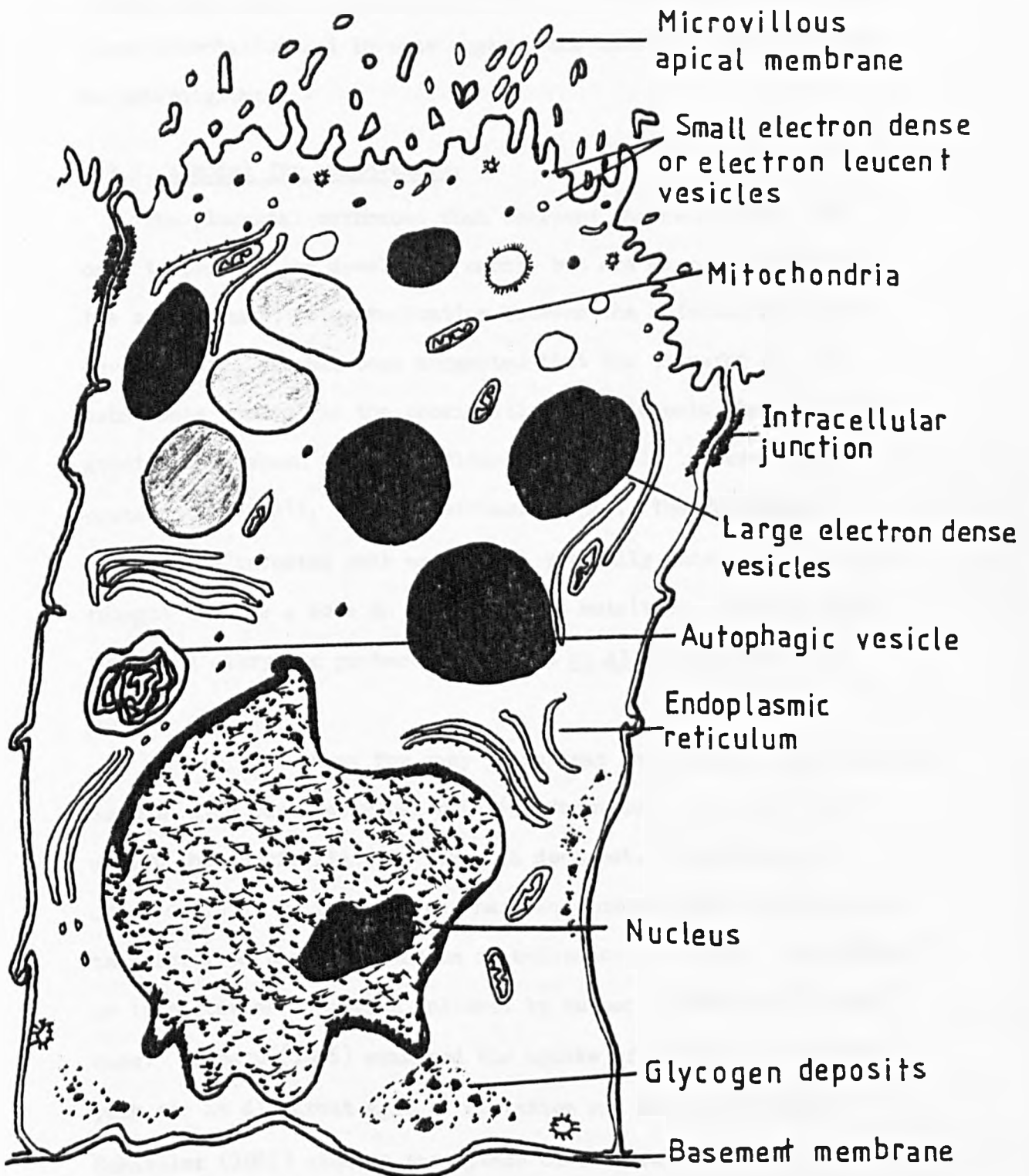




FIG.1.4. AN ELECTRON MICROGRAPH OF A TYPICAL
YOLK SAC EPITHELIAL CELL. (stained with
lead citrate/uranyl acetate) magnification x 11,500

FIG. 1.5. A TYPICAL YOLK SAC EPITHELIAL CELL



the nucleus and the basal plasma membrane. Numerous mitochondria are located throughout the cell, the larger ones particularly in close association with strands of rough endoplasmic reticulum just beneath the apical canalicular system. Adjacent cells are in very close association and in some regions the lateral membranes appear to interdigitate.

1.3.2. A model for pinocytosis:

The placental membranes that surround the fetus serve not only to protect the developing embryo but are also concerned with the maintenance of communication between the maternal and fetal environments. It has been suggested that the transfer of most substances occurs via the chorioallantoic placenta, but several studies have shown that this tissue is probably impermeable to protein (Brambell, 1958; Mayersbach, 1958). The functional role of the inverted yolk sac is not yet fully understood. It is thought to have a role in embryotrophic nutrition, (Beck & Lloyd 1968) and embryonic protection (Wilson et al., 1959; Brambell, 1970).

It has been known for many years that the yolk sac can accumulate certain dyes such as trypan blue (Goldman, 1909, Everett, 1935) whilst the chorioallantoic placenta does not. The uptake of other colloids by the yolk sac has been investigated using methods that involved the introduction of substances by either intravenous or intrauterine injection followed by subsequent removal of yolk sacs. Lambson (1966) examined the uptake of ferritin by the rat yolk sac at different ages of gestation and Krzyzowska-Gruca & Schiebler (1967) studied the uptake of both ferritin and trypan blue

by the rat yolk sac. Luse (1957) suggested that large macromolecules are taken up from the uterine cavity by a mechanism of pinocytosis and more recent electron microscopical visualization of such processes confirm this.

Over the last ten years Lloyd and co-workers have developed a quantitative technique for the measurement of pinocytosis in the rat visceral yolk sac. An early interest was the degradative capacity of the yolk sac with regard to its role in embryotrophic nutrition. This was investigated by intravenous injection of horseradish peroxidase into pregnant rats, followed by histochemical and biochemical investigations of the embryo and its membranes (Beck et al., 1967). Later explants of the mesometrial pole of yolk sacs were taken following intravenous injection of horseradish peroxidase and then cultured using the raft technique originally described by Trowell (1959), (Beck et al., 1970). This series of experiments showed that horseradish peroxidase was taken up by the yolk sac cells and to some extent degraded, but confirmed that no intact peroxidase reached the embryo. To study the kinetics of the pinocytic process in more detail, an organ culture technique was devised (Williams et al., 1975 a,b), whereby the pinocytic capture of both non-degradable and degradable macromolecules could be measured. The methodology of this technique and subsequent modifications are described in detail in Chapter 2 but an introduction to the terminology used for quantitation is of value here. Williams et al., (1975 a,b) introduced the term "Endocytic Index" to describe the rate of capture of macromolecules by the rat yolk sac, the Endocytic Index being defined as the volume of culture medium whose contained substrate is captured per mg of yolk sac protein per hour. Expression

of substrate uptake in these terms has several advantages, the most important being the ability to compare results between experiments both with the same, and different substrates, allowances being made for any differences in the specific activity of the substrate used.

The yolk sac technique has been used to study many aspects of pinocytosis in this tissue. Initially a non-degradable macromolecule, ^{125}I -labelled polyvinyl pyrrolidone (^{125}I -PVP) was used as a marker (Williams et al., 1975 a) and the mean Endocytic Index for this substrate was $1.71 \mu\text{l/mg protein/h}$. More recently [^{14}C]sucrose has also been found to be captured at a similar rate, having a mean Endocytic Index of $2.04 \mu\text{l/mg protein/h}$ (Roberts et al., 1977). In contrast colloidal ^{198}Au and ^{125}I -labelled denatured bovine serum albumin (^{125}I -dBSA) have higher Endocytic Indices, approximately 3.5 and $60 \mu\text{l/mg protein/h}$ respectively (Roberts et al., 1977; Williams et al., 1975 b) but these substrates showed a marked batch variation in the rate of uptake.

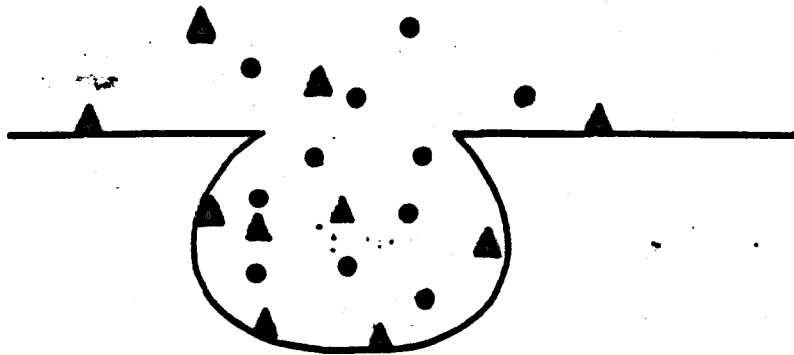
Jacques (1969) suggested two mechanisms by which macromolecules are taken up during pinocytosis and these involve the two phases involved during vesicle formation; the plasma membrane that forms the boundary of the vesicle and also any fluid that is encapsulated therein. Firstly molecules may be taken up in solution and this is commonly called "fluid-phase or bulk-phase pinocytosis" or secondly they may adsorb to the plasma membrane and be carried inwards during invagination, commonly called "adsorptive pinocytosis". Fig.1.6. shows the three theoretical models that could be proposed to explain the mechanism of capture of any substrate, and they are as follows:-

1. A substrate with no affinity for the plasma membrane could be captured entirely in the fluid phase.

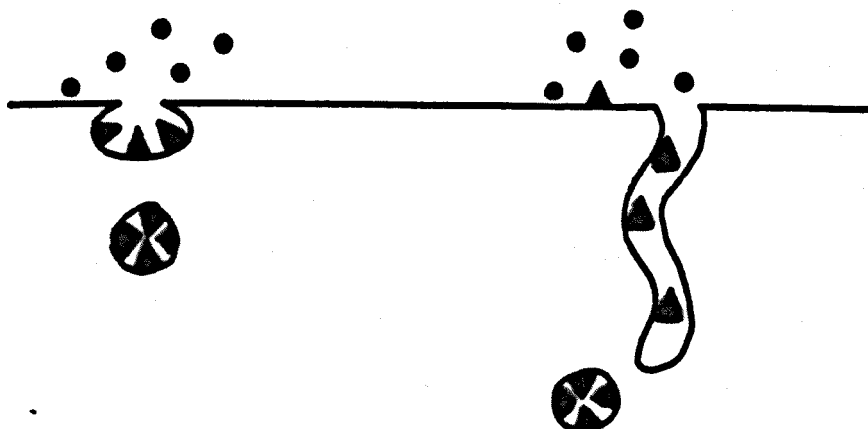
FIG.1.6. THEORETICAL MECHANISMS OF
SUBSTRATE CAPTURE



i) entry entirely in the fluid-phase



ii) entry by both adsorptive & fluid-phase pinocytosis



iii) selective entry of adsorbed substrate

2. A substrate with affinity for the membrane could be captured in association with the membrane and in the fluid phase.
3. With selectivity at the plasma membrane a bound substrate could be selected specifically with total exclusion of fluid.

Certain criteria may be used to establish whether the mode of uptake of a substrate is adsorptive or fluid phase and these are discussed extensively by Pratten et al., (1979).

Briefly they may be outlined as follows:-

i) The magnitude of the calculated Endocytic Index for a substrate can give a good indication of the mechanism of capture. It may be found that one or more substrates enter a system at the same (low) rate, which is less than that obtained for any other substrate. It is usually postulated that uptake of these substrates is entirely fluid-phase. Deviation from the established fluid phase rate of uptake can have several explanations. A higher Endocytic Index is most likely to be a function of the degree of membrane adsorption, but would also result if the substrate was stimulating pinosome formation at the plasma membrane. Likewise a lower Endocytic Index would suggest the established fluid-phase rate was invalid, unless the substrate could inhibit the rate of membrane invagination. These alternatives can be distinguished by assessing the effect of such substrates on the uptake of an accepted fluid phase marker.

ii) Increasing the extracellular concentration of a substrate that enters entirely in the fluid phase is without effect on the rate of uptake expressed in terms of $\mu\text{l}/\text{mg}$ protein/h, but uptake in terms of ng of substrate internalized increases linearly with

concentration. A substrate entering by an adsorptive mechanism does so in a concentration-dependent manner; as the concentration increases the membrane binding sites become saturated with substrate and as a result the Endocytic Index tends towards the fluid phase value.

iii) When cells are ingesting substrate that enters to a large extent in a membrane-bound fashion are incubated with the substrate and then subsequently incubated in substrate-free medium, any externally bound material tends to be released into the culture medium. Any internalized material can only be released by an exocytic mechanism and so a fluid-phase marker is only released by this route. Thus if the tissue association of a substrate has a readily reversible component, it can be indicative of an adsorptive mechanism. Release of large quantities of material by trypsin treatment is useful in determining the extent of membrane association of a substrate.

iv) Inhibitors, such as those which disrupt the metabolic energy supply or the cytoskeletal system, can be used to block endocytic uptake. The quantity of substrate associated with the system under conditions of inhibited membrane internalization can be used to assess the importance of a binding component to the normal progressive uptake process.

In the rat visceral yolk sac pinocytosis of ^{125}I -PVP and $[^{14}\text{C}]$ sucrose is thought to represent fluid-phase uptake only with no, or minimal membrane adsorption (Roberts et al., 1977). Although it has been suggested that native protein and colloidal ^{198}Au might also enter this tissue in the fluid phase alone (Lloyd, 1977.a; Lloyd & Williams, 1978), it is now apparent that both these substrates and to a greater extent denatured proteins also enter the cell by

an adsorptive mechanism. Neither colloidal ^{198}Au nor ^{125}I -dBSA stimulate vesicle formation in this tissue (as assessed by their inability to stimulate the uptake of ^{125}I -PVP) so it is suggested that their high Endocytic Indices simply reflect their ability to bind to the yolk sac membrane thus entering more rapidly.

1.4. AIMS OF CURRENT STUDY.

It is obvious from the first section of this Introduction that most of the knowledge relating to the endocytic mechanism is confined to phagocytosis, there being little controversy over the role of the attachment phase, and the mechanism of and prerequisites for internalization. In fact the current status of our understanding of phagocytosis was recently reviewed in an amusing article entitled "How do phagocytes eat?" (Stossel, 1978). In contrast, the underlying mechanisms of pinocytosis are still obscure and the intricacies of this process remain largely unexamined. There are certain aspects of pinocytosis that urgently require detailed investigation. It is known that macromolecules captured pinocytically are taken up either in solution or in association with the plasma membrane, but the determinants for high membrane affinity and their cellular specificity are not fully understood for many substrates. Such considerations are of particular importance if one wishes to direct pinocytic substrates to a particular cell type where they can be internalized efficiently and subsequently initiate a pharmacological response. An alternative method for the enhancement of the intracellular concentrations of pinocytic substrates would be to stimulate the rate at which a cell forms pinosomes at the plasma membrane. To achieve this we must discover factors which are responsible for the control of pinocytic rate in the cell. Could pinocytosis simply be an intrinsic cell function, which once instituted is an ongoing mechanism subjected to no fine rate control? Are there different types of pinocytosis, some mechanisms which are subject to rate control, others that are not? As yet there are no answers to these questions although it has been suggested that pinocytic processes

can be divided into categories depending on their energy requirements, sensitivity to cytoskeletal inhibitors and also the vesicle size involved. The validity of such divisions is arguable, but in order to propose a better scheme there is a pressing need to gather quantitative information on many pinocytic processes in a wide variety of cell types.

Over the past fifteen years attempts to understand pinocytosis have been severely hindered by the lack of reliable quantitative methods for assessing pinocytic activity and also the absence of dependable model systems. Recently both of these problems have been overcome with the development of the concept of Endocytic Index as a measure of the clearance of pinocytic substrates and also the discovery that the rat visceral yolk sac is an extremely effective pinocytic system. These techniques are used in this thesis to examine quantitatively some of the questions outlined above. The work falls into two main parts:-

i) an investigation of some factors that might be important in the control of pinocytic rate in the rat visceral yolk sac, such as temperature, metabolic inhibitors, cytoskeletal inhibitors (Chapter 4) and factors which have been shown to modify endocytosis in other systems, such as polyaminoacids and dextrans (Chapter 5) and DIVEMAs (Chapter 8). The mechanism of action of some of these modifiers is examined (Chapters 6 and 7).

ii) a study of effect of the physical size of substrate on its rate of pinocytosis by the rat visceral yolk sac (Chapter 10). This study was carried out with polymers of precisely defined molecular weight distribution and the development of a suitable procedure of radiolabelling was undertaken (Chapter 9).

CHAPTER 2

MATERIALS AND GENERAL METHODS.

2.1. MATERIALS

2.1.1. Chemicals.

^{125}I -PVP (preparation IM.33P)

[^{125}I] Iodide (preparation IMS.4)

Colloidal ^{198}Au (preparation GCS.1P)

Radiochemical Centre,
Amersham, Bucks.

Tissue culture medium (TC 20)
Heat inactivated calf serum (CS07)

Wellcome Reagents,
Beckenham, Kent.

Bovine serum albumin

2,4 - Dinitrophenol

Colchicine

Monoiodoacetic acid

Cytochalasin B

Theophylline

Ouabain

EGTA

Poly-L-ornithine (M_r 100,000 - 200,00)

Poly-L-lysine ($M_r > 70,000$)

Poly-L-glutamic acid (M_r 50,000 - 100,000)

DEAE-dextran (Average M.wt. 500,000)

Dextran sulphate (Average M.wt. 500,000)

Dextran (Average M.wt. 500,000)

PVP (Average M.wt. 40,000)

Sigma (London) Chemical
Company, Poole, Dorset.

Sephadex G-25-80

Sephadex G-10-120

Bovine serum albumin
(preparation 0142t)

Koch-light Laboratories,
Colnbrook, Bucks.

Oxygen/Carbon dioxide (95:5)

British Oxygen Co Ltd.
Manchester.

Zerolit FF1P (SRA 66)

BDH Chemicals Ltd.
Poole, Dorset.

DIVEMAs and DIVEMA derivatives
PVP (preparations S1 - S4)

Kindly donated by Prof.
H. Ringsdorf, University
of Mainz, W. Germany.

2.1.2. Equipment.

Assay of Radioactivity

5142 Selektronic Gamma Spectrometer

Packard Instrument Ltd.
Caversham, Berks.

3ml Disposable Tubes

Luckhams Ltd, Burgess
Hill, Sussex.

Centrifuge

MSE Mistral 4L

MSE Instruments,
Crawley, Sussex.

Electrophoresis Equipment

Shandon Electrophoresis Apparatus
Model 477

Shandon Scientific
Company Ltd. Willesden,
London.

Chandos Power Pack
Model No E 26

Chandos, Stockport.

Spectrophotometer

Cecil CE 273

Cecil Instruments Ltd.,
Milton, Cambs.

2.2. RAT VISCERAL YOLK SAC CULTURE TECHNIQUE

A method for culturing the rat yolk sac has been previously described (Williams et al., 1975 a,b) and the general procedure was followed throughout with some minor modifications. An inbred colony of Wistar rats were used to obtain yolk sacs of small genetic variation. Animals were mated overnight and then, if a sperm plug was detectable underneath the grid of the cage the following morning, pregnancy was timed from midnight on the night of mating. After 17.5 days the rats were killed by either cervical dislocation or carbon dioxide poisoning and the uterus removed and placed in a petri dish containing medium 199 with 10% calf serum at 37°C. By a single longitudinal cut through the uterine wall the conceptuses were exposed and then the placental cap of each conceptus was prised free using open fine scissors. Again using fine scissors the placental caps were removed, taking care to minimize damage to the yolk sac tissue, and the cut edge of the yolk sacs enlarged by a single cut. The yolk sac was dissected free from the fetuses and placed in another petri dish containing fresh medium. There the (more transparent) amnion was very gently pulled away from the yolk sac taking care to preserve yolk sac integrity. Its removal is relatively easy, so this is always undertaken; however caution is required as any damage incurred by the yolk sac during the procedure could be more disadvantageous than the retention of a small portion of amnion. Total dissection time should be as short as possible and certainly less than 15 min. A correlation has been found between increased dissection time

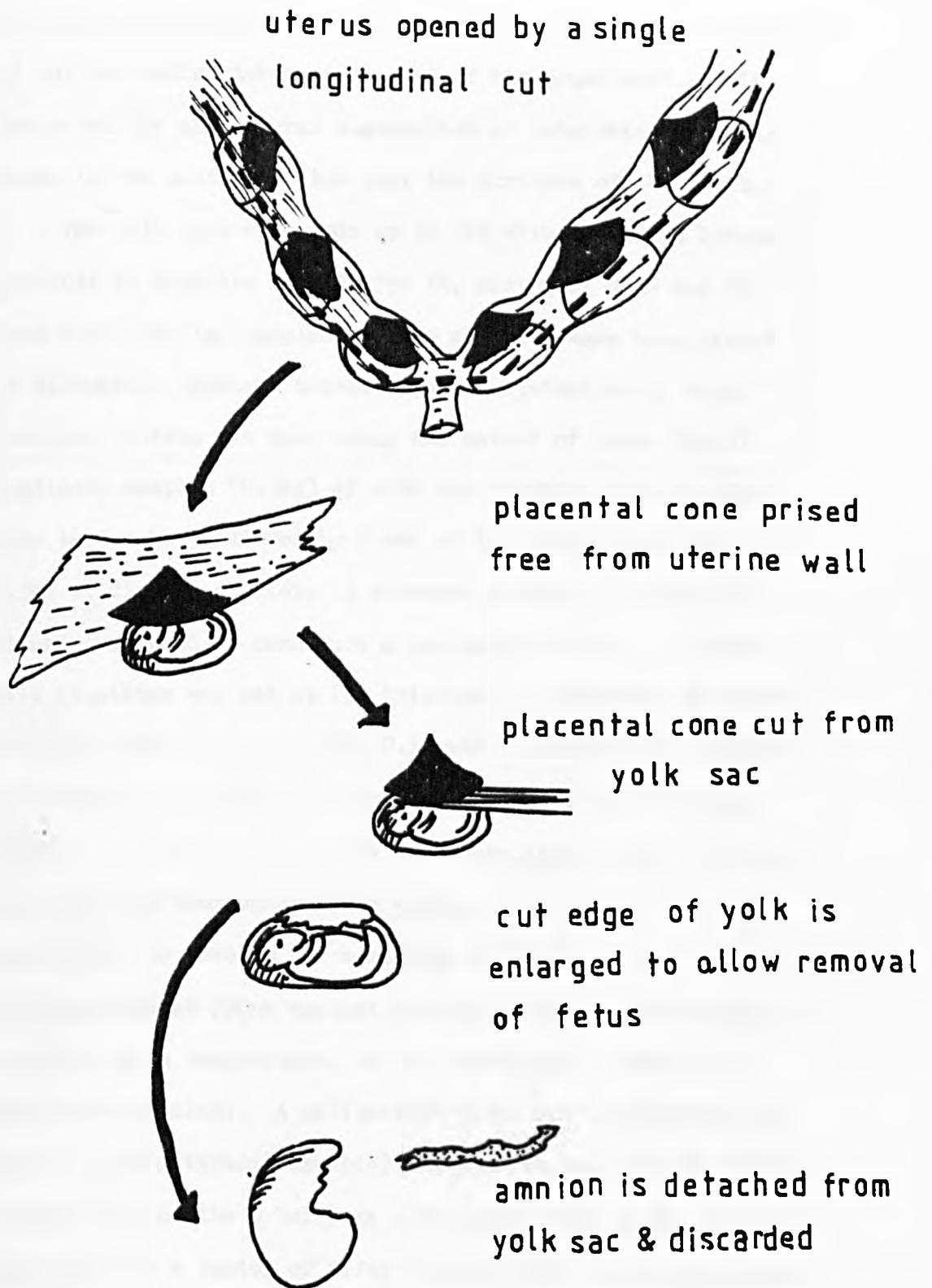
and decreased Endocytic Index, which is believed to reflect a decrease in cellular survival time (Williams unpublished data). Fig. 2.1.gives diagrammatic representation of the general dissection procedure.

The yolk sacs were incubated in sterile 50ml Erlenmeyer flasks containing 10ml of medium 199 and 10% calf serum. The flasks were gassed with a mixture of oxygen and carbon dioxide (95:5) both before the introduction of a yolk sac and after addition of substrate, and were sealed by means of a sterile silicone rubber bung. They were maintained in a constant temperature water bath preset to 37°C with a shaker attachment regulated to an amplitude of shake of approximately 3.5cm and a frequency of shake of approximately 100 strokes/min. Smoothness in the action of the shaker attachment is essential as 'snatching' may damage the yolk sac in culture.

Usually 9ml of culture medium (containing 10% calf serum) was added to the flask before the introduction of a yolk sac so that the substrate could be dispensed in 1ml aliquots of culture medium. Before the addition of substrate yolk sacs were preincubated for a period of approximately 15 min so that the tissue could reach steady state.

Incubations were routinely carried out for 6-7h and at equally spaced intervals a yolk sac was taken from its flask and washed (3 x 2 min) in approximately 20ml of ice-cold 1% sodium chloride and then transferred to a 5ml volumetric flask. Duplicate 1ml samples of culture medium were taken from each flask and placed in disposable Luckhams tubes. In cultures

FIG. 2.1. GENERAL DISSECTION PROCEDURE



that involved the use of a degradable substrate the flasks were returned to the water bath after each sampling and the samples of culture medium taken at the end of the experiment. This accounted for any natural degradation of substrate which may occur in the culture medium over the duration of the incubation.

The yolk sacs were made up to 5ml with 1N sodium hydroxide and left to dissolve at 37°C for 1h, mixing at 0.5h and 1h. Then duplicate 1ml samples of this solution were transferred to disposable Luckhams tubes. Also an estimation of total yolk sac protein was made using the method of Lowry (1951). Duplicate samples (0.1ml) of yolk sac solution were pipetted into test tubes followed by 0.4ml of 1N sodium hydroxide and 0.5ml of distilled water. A standard solution (1mg/ml) of albumin was used to construct a calibration curve. A series of five dilutions was set up (in triplicate) consisting of albumin concentrations 0, 0.05, 0.10, 0.15 and 0.20 mg/ml and solutions were added to the tubes as described in Table 2.1. Folin A (5.0ml) was added to each tube and 20min later 0.5ml of Folin B was added and thoroughly mixed using a Whirlimix. The colour was allowed to develop for a minimum of 45min at room temperature and then read at 750nm against distilled water. (The colour is stable at room temperature, so the tubes can be left up to 24h before reading). A calibration curve was constructed (see Fig.2.2. for a typical example) and used to read off the protein content (mg) of the 0.1ml yolk sac sample. The values were then multiplied by a factor of fifty to give total yolk sac protein.

TABLE 2.1. ADDITIONS TO THE CALIBRATION CURVE FOR LOWRY
PROTEIN ESTIMATION

| <u>Tube</u> | <u>Protein Standard (mg/ml) (ml)</u> | <u>Distilled Water (ml)</u> | <u>IN Sodium Hydroxide (ml)</u> |
|-------------|--|---------------------------------|-------------------------------------|
| 1 | 0 | 0.5 | 0.5 |
| 2 | 0.05 | 0.45 | 0.5 |
| 3 | 0.10 | 0.40 | 0.5 |
| 4 | 0.15 | 0.35 | 0.5 |
| 5 | 0.20 | 0.30 | 0.5 |

FOLINS REAGENTS

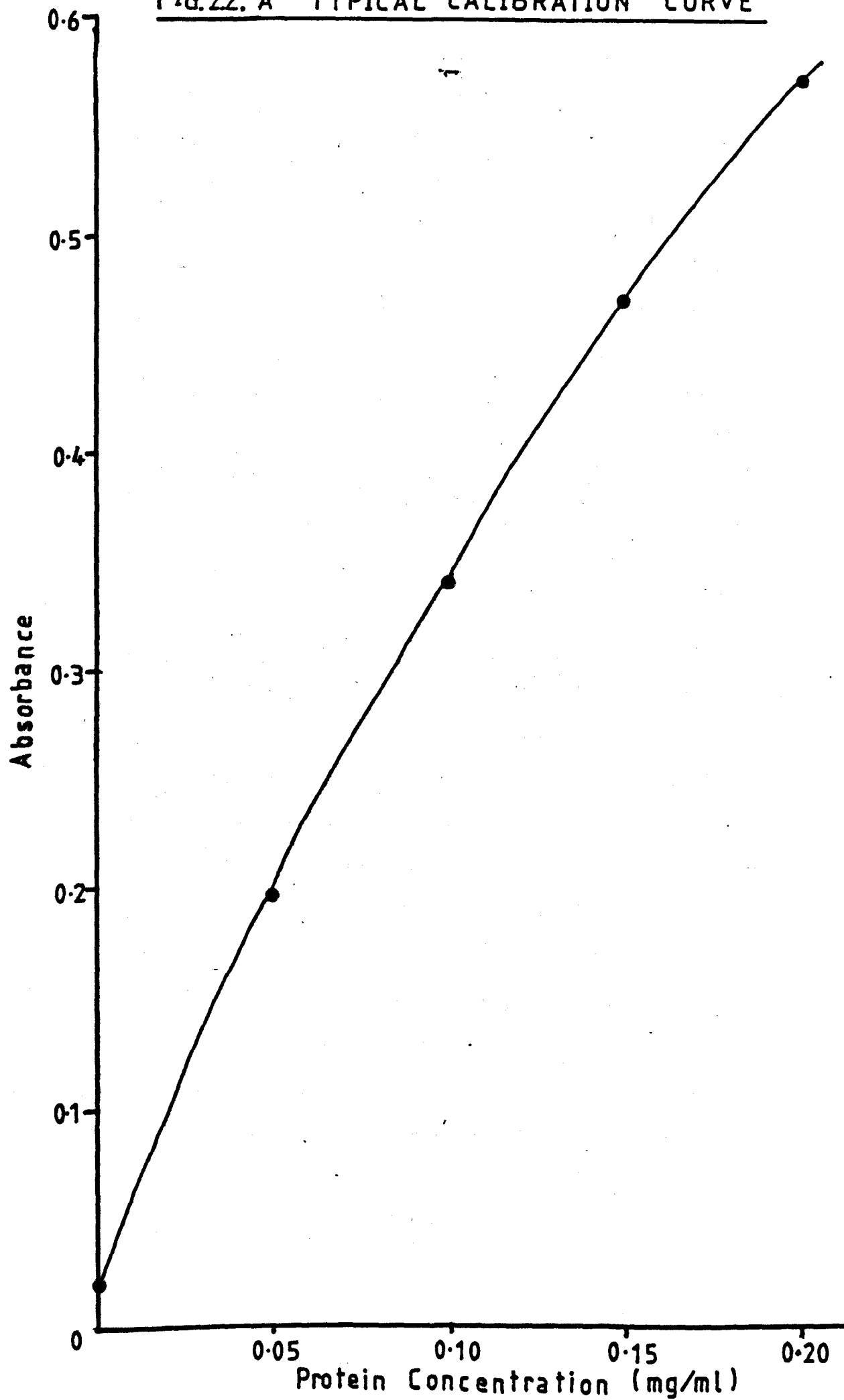
FOLIN A

2% anhydrous sodium carbonate 100ml
1% copper sulphate 1.0ml
1% sodium, potassium
tartrate 1.0ml

FOLIN B

Folin Ciocalteu's reagent 1ml.
Distilled water 1ml.

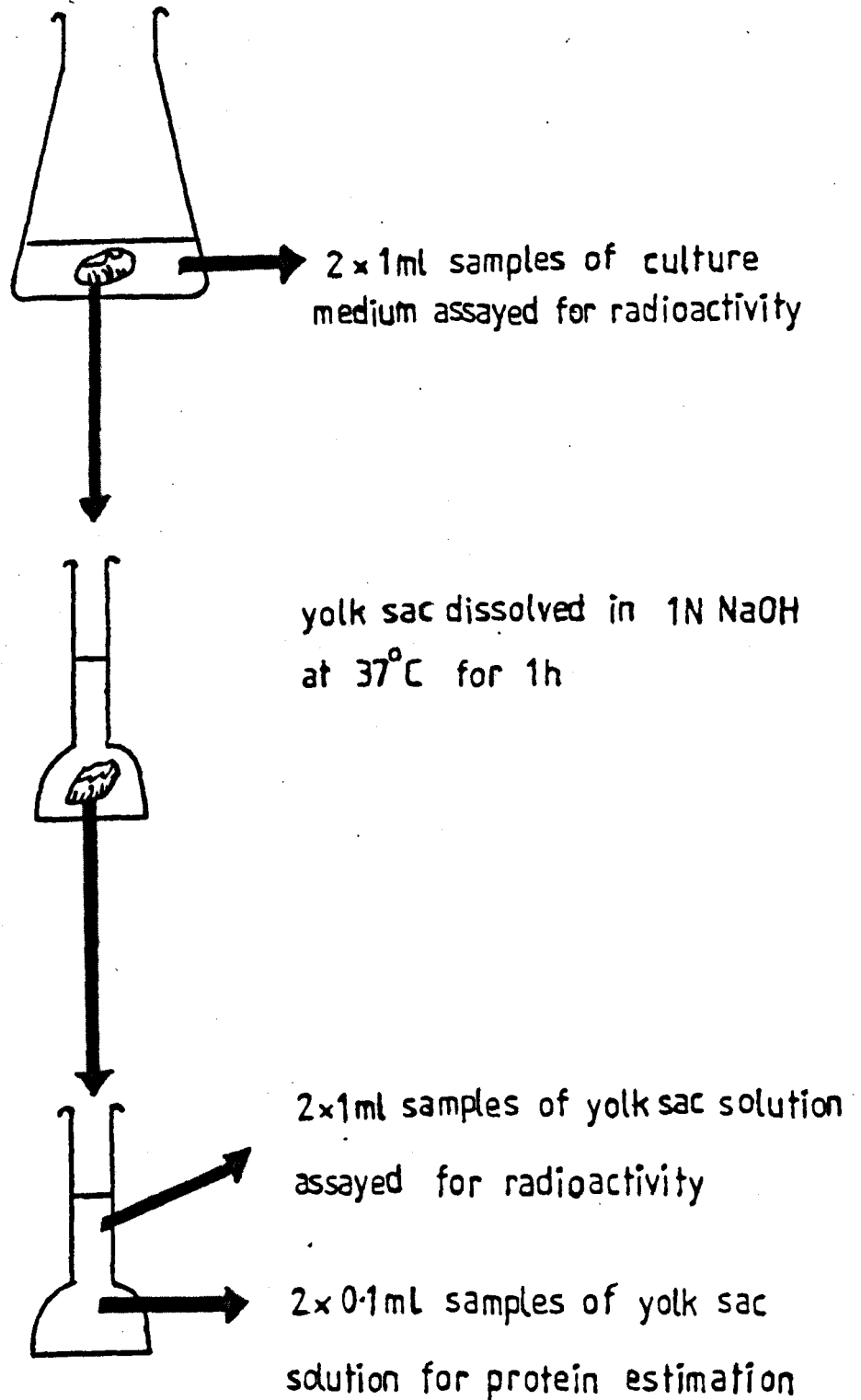
FIG.22. A TYPICAL CALIBRATION CURVE



Over the course of many protein estimations some variation in the calibration curve was observed but this was relatively small. The optical densities of the uppermost point usually fell in the range 0.55 to 0.65 and the relationship between protein and absorbance was always non-linear. The slight variations were attributed to small discrepancies in pipetting, making up the protein standard or concentrations of the reagents used for the assay. Only a large inaccuracy in the concentration of the protein standard would seriously influence the protein estimation. Other variables (automatic pipetting devices were used throughout) were common to both the experimental tubes and the standard curve and hence their affect would be nullified. The general culture procedure is summarized in Fig.2.3.

In some experiments yolk sacs were incubated in medium 199 without calf serum. This technique has been described by Ibbotson & Williams (1979) and the general methodology differs little from the technique described above. In the absence of serum dissection time would seem even more vital and, to obtain optimum speed, ideally two persons were required. Ibbotson (1978) and subsequent investigators of this system (Livesey, 1979, Weisbecker, unpublished results) observed a decreased linearity in their data after approximately 5h of culture; however, to maintain uniformity between experiments, the no-calf serum cultures undertaken here were run for the usual 6-7h and all the data analysed with no indiscriminate removal of any points which deviated from a general trend. This may have increased

FIG.23 GENERAL CULTURE PROCEDURE



the scatter about the regression line (i.e decreased the correlation coefficient, see Section 2.11.) but certainly did not bias the data in any way.

2.3 QUANTITATION OF THE UPTAKE OF A NON-DEGRADABLE SUBSTRATE.

Two non-degradable substrates were used in this study, ^{125}I -PVP and colloidal ^{198}Au and they were usually used at concentrations of 2 $\mu\text{g/ml}$ and 1 $\mu\text{g/ml}$ respectively. The procedure for quantitation is the same for both. Following incubation 1ml aliquots of yolk sac solution, and the corresponding portions of culture media, were assayed for radioactivity with a gamma counter and the total yolk sac protein estimated (see Section 2.2.). To calculate uptake at each time interval in terms of the volume of culture medium whose contained substrate is captured per mg yolk sac protein, the following equation was used:-

$$U = \frac{Y}{M \times P}$$

where U is uptake ($\mu\text{l/mg}$ protein)

Y is total radioactivity (counts per min corrected for background)

M is radioactivity per μl of culture medium (counts per min corrected for background)

P is protein content (mg)

In practice this calculation was speeded up by using the simple computer programme shown in Appendix I.

The unmodified uptake of the substrates used was consistently linear with time and the rate of uptake of the substrate was derived from the plot of uptake against time, using a best fit regression line to describe the relationship. Routinely a correlation coefficient was also estimated. In all cases the rate of uptake was termed an Endocytic Index, the units being $\mu\text{l/mg}$ protein/h.

In experiments where uptake is non-linear with time (either due

to addition of a modifier or for any other reason), it is not possible to estimate an Endocytic Index, so it is very important to assess the degree of linearity of the uptake points before haphazardly applying regression analysis: the regression coefficient would be meaningless. In these cases the uptake/time plots are used to describe any effects and for a series of experiments the mean uptake at each time interval is calculated.

2.4. QUANTITATION OF THE UPTAKE OF A DEGRADABLE SUBSTRATE.

^{125}I -dBSA was the only degradable substrate investigated and was used at a concentration of $1\text{ }\mu\text{g/ml}$. Samples of yolk sac solution and the corresponding portions of culture medium were assayed for radioactivity with a gamma counter and the total yolk sac protein estimated (see Section 2.2.). Trichloroacetic acid (TCA, 20% wt/vol, 0.5ml) was added to the tubes containing culture medium and then they were centrifuged at 2,000g for 20min. The supernatant was then decanted into a fresh tube and assayed for radioactivity. Throughout a standard volume of 1ml was used for assay of radioactivity in culture medium and yolk sac solution, but addition of TCA increased this volume.

The observed values for radioactivity within these samples were multiplied by a correction factor and this constant value compensated for the disparity introduced by the following two factors during the precipitation procedure:-

- i) Increasing the standard volume of 1ml by addition of 0.5ml of TCA resulted in a post-centrifugation supernatant volume of approximately 1.3ml. The resultant change in counting geometry alters the counting efficiency of the gamma counter.
- ii) Precipitation of the macromolecular ^{125}I -dBSA also caused a small degree of "trapping" of the TCA-soluble digestion products ^{125}I -tyrosine and glycyl- ^{125}I -tyrosine (these were identified as the digestion products of ^{125}I -dBSA by Williams et al., 1975 b).

A correction factor which reconciles both of these effects was simply estimated as follows. ^{125}I -tyrosine and glycyl- ^{125}I -tyrosine were diluted in medium 199 containing 10% calf serum to produce an acceptable number of cpm. The three series of Luckhams tubes (6 in each series) were set up as follows:-

A. 1ml of ^{125}I -tyrosine

B. 1ml of glycyl- ^{125}I -tyrosine

C. 0.5ml of ^{125}I -tyrosine and 0.5ml of glycyl- ^{125}I -tyrosine

These tubes were then counted for 30s, 0.5ml of TCA (20% wt/vol) was added, centrifuged in the usual manner and the supernatant recounted. A correction factor for each sample was calculated as follows:-

$$\text{Correction Factor} = \frac{\text{Total counts} - \text{Background}^+}{\text{TCA soluble counts} - \text{Background}^+}$$

⁺Estimated over the same period

The results are summarized in Table 2.3. and the correction factor used for subsequent experiments was 1.3.

To calculate any TCA-soluble radioactivity present within a preparation of ^{125}I -dBSA, or alternatively released during the incubation period, a control was always included which consisted of a flask containing medium 199 (and 10% calf serum) together with substrate, and this remained in the water bath for the duration of the culture. This initial soluble value was usually in the order of 1% and the medium TCA-soluble counts were corrected accordingly.

The uptake of ^{125}I -dBSA in terms of volume of culture medium whose contained substrate is captured per mg yolk sac

TABLE 2.2. ESTIMATION OF A CORRECTION FACTOR FOR ASSAY OF TCA
SOLUBLE RADIOACTIVITY FOR THE QUANTITATION OF THE
UPTAKE OF DEGRADABLE SUBSTRATE.

| Marker | | | |
|--------------------|----------------------------|------------------------------------|---|
| Replicate | ^{125}I -tyrosine | glycyl- ^{125}I -tyrosine | ^{125}I -tyrosine/ glycyl- ^{125}I -tyrosine |
| 1 | 1.296 | 1.310 | 1.319 |
| 2 | 1.311 | 1.265 | 1.314 |
| 3 | 1.282 | 1.315 | 1.376 |
| 4 | 1.330 | 1.245 | 1.300 |
| 5 | 1.284 | 1.271 | 1.329 |
| 6 | 1.273 | 1.297 | 1.335 |
| Mean \pm S.E. | 1.296 \pm 0.009 | 1.280 \pm 0.011 | 1.329 \pm 0.011 |

Overall Mean 1.29

protein was calculated using the following equation:-

$$U = \frac{Y + 10 (S-F)}{M' \times P}$$

Where U is uptake (μ l/mg protein)

Y is total radioactivity (counts per min corrected for background)

M' is TCA-insoluble radioactivity in the culture medium (counts per minute per μ l medium corrected for background)

P is protein content (mg)

S is TCA-soluble radioactivity in the culture medium at the end of an incubation period (counts per min per ml medium corrected for background)

F is TCA-soluble material in the preparation at the beginning of incubation and that released by spontaneous degradation during culture

In this equation the component relating to "TCA-insoluble" medium radioactivity is designated M'. Many proteins including 125 I-dBSA are pinocytosed much more rapidly than 125 I-PVP and this leads to appreciable depletion in the concentration of substrate in the medium over the culture period. To compensate for this effect a corrected value for "TCA-insoluble" radioactivity (cpm per ml) is calculated by adding back to the measured "TCA soluble" activity (cpm per ml) half the increase in "TCA soluble" activity (cpm per ml) measured at the time of sampling. Thus the mean quantity of "TCA-insoluble" material available for capture by the yolk sac over the incubation period is used for estimation of uptake.

$$M' = M + \frac{(S - F)}{2}$$

In practice the calculation of uptake was speeded up by using the computer programme shown in Appendix II.

2.5. QUANTITATION OF EXOCYTOSIS OF NON-DEGRADABLE SUBSTRATES.

To estimate the rate of release of a non-degradable substrate the following method was used. Yolk sacs were incubated for 3h in 10ml of medium 199 containing 10% calf serum and radiolabelled substrate. They were then washed (3 x 2min) in approximately 20ml of medium 199 and 10% calf serum at 37°C and transferred to 10ml of medium that had been gassed and warmed to 37°C and contained an equal quantity of unlabelled substrate. Incubation was continued for 3h and duplicate 1ml samples of medium were removed at equally spaced intervals throughout this time. After each sample the medium withdrawn was replaced by an equal volume of fresh medium which had been warmed to 37°C and gassed. Throughout the incubation the flask itself was regassed at regular intervals.

In some experiments modifiers were included in the incubation medium, either during the preincubation phase, reincubation phase or at both stages.

The radioactivity remaining in the yolk sac at the end of the experiment was estimated as described in Section 2.3. To calculate the total radioactivity taken up by the yolk sac over the first 3h of incubation, the activity released during the exocytosis phase was summed, making allowances for the changing volume effects and correcting for background, and then added to the measured tissue level remaining at the end of this time (see Table 2.3. for a typical example). Release of radioactivity at each time interval was then expressed as a percentage of the total and plotted against time. Over the linear portion of the graph the rate of release (percentage per hour) was calculated.

TABLE 2.3. A TYPICAL EXOCYTOSIS EXPERIMENT: DATA HANDLING

| Incubation Time (min) | Medium Radioactivity (corrected for background) | | | Exocytosed | Release % of initial radioactivity |
|--------------------------|---|------------------|-------------------|------------|--|
| | cp 5m per ml | cp 5m per 2ml | cp 5m per 10ml | | |
| 15 | 37.3 | 74.6 | 373 | 373 | 4.35 |
| 30 | 38.5 | 77.0 | 385 | 460 | 5.37 |
| 45 | 27.2 | 54.4 | 272 | 424 | 4.95 |
| 60 | 36.2 | 72.4 | 362 | 568 | 6.63 |
| 120 | 55.5 | 111.0 | 555 | 833 | 9.72 |
| 180 | 51.3 | 102.6 | 513 | 902 | 10.53 |

Total yolk sac radioactivity (corrected for background)

$$= 8571 \text{ (cp 5m)}$$

The activity released at each time interval was calculated using this equation:-

$$\text{Total activity} = 10 \times (\text{Medium activity per ml of sample } n) + \sum_{0}^{n-1} 2 \times (\text{Medium activity per ml of sample})$$

The total yolk sac radioactivity was determined using the following equation:-

$$\text{Total Uptake} = \text{Yolk sac radioactivity after release phase} + \text{Total radioactivity released into culture medium.}$$

2.6. ESTIMATION OF THE UPTAKE OF ^{125}I -PVP AFTER EXPOSURE OF THE YOLK SAC TO VARIOUS MODIFIERS

These were termed "recovery experiments" and were performed to test the ability of the yolk sac to resume the normal rate of pinocytosis after exposure to a variety of inhibitors. Incubations were carried out for 3.5h in 10ml of medium 199 and 10% calf serum containing ^{125}I -PVP and an inhibitor, or alternatively ^{125}I -PVP alone, samples being taken at regular intervals during this period. The remaining yolk sacs were removed and washed (3 x 2 min) in medium at 37°C and then transferred to culture flasks containing an equivalent volume of warm, gassed culture medium, no inhibitor, and exactly the same concentration/specific activity of ^{125}I -PVP. This was achieved by dispensing ^{125}I -PVP and modifier separately and using aliquots of ^{125}I -PVP from the stock solution for all flasks. Samples were taken over the remaining 3.5h of the culture period. Uptake was calculated as described in Section 2.3. and values ($\mu\text{l}/\text{mg}$ protein) were plotted against time. An Endocytic Index was calculated for the values obtained, both in the presence and absence of inhibitor, and similarly Endocytic Indices were found for the control data where the only differences in the two incubation phases was that the yolk sacs used in the latter part of the experiment had been subjected to the washing procedure.

2.7. PREPARATION OF ^{125}I -dBSA

^{125}I -dBSA was prepared according to the Chloramine T method originally described by Hunter et al., (1963). The modification used by Williams et al., (1971) was followed here. Bovine serum albumin (BSA) was dissolved in 9.0ml of phosphate buffer (Na_2HPO_4 - KH_2PO_4 12H₂O-7.098g per litre) at pH 8.0 and cooled to 4°C. [^{125}I]iodide (1mCi) was added and the mixture stirred for 2 min. Then 4ml of chloramine T (1mg/ml solution) was added and stirred for 8.0min. The reaction was stopped with 3.0ml of a sodium metabisulphite (2mg/ml solution) and again stirred for 2.0min. Solid potassium iodide (100mg) was added to displace free ^{125}I iodide during the subsequent dialysis. An equal volume of formaldehyde solution (250ml of 40% formalin and 52.99g of Na_2CO_3 made up to a litre with distilled water and titrated to pH10 with 5N HCl) was added and the reaction mixture left to stand at 4°C for 72h. This was dialysed against 5 litres of 1% sodium chloride for 3-4 days with two daily changes of the sodium chloride solution. The percentage TCA soluble radioactivity was estimated for each batch before use and a level of less than 2% was deemed acceptable.

2.8. ELECTROPHORESIS: A METHOD OF ESTIMATION OF $[^{125}\text{I}]$ IODIDE
IN A PREPARATION OF ^{125}I -PVP

Low voltage paper electrophoresis proved to be a useful method for the estimation of free $[^{125}\text{I}]$ iodide in a preparation of ^{125}I -labelled substrate. There are many texts which describe this technique; Sargent (1969) was followed. Whatman Nol paper was cut into strips about 30cm in length and 10cm wide. The central 20cm was pencil marked into 40 x 0.5cm strips and in the fifth strip from one end, the origin marked. Barbitone buffer, pH 8.6 was prepared (1.84g of barbitone and 10.30g of barbitone sodium in 1 litre of distilled water) and the electrophoresis tank filled to a depth of approximately 2cm. The papers (usually four strips were run at any time) were then dipped in the barbitone buffer and blotted lightly between sheets of filter paper. The damp papers were placed across the supporting bars in the electrophoresis tank so that the origin was nearest the cathode (this was not of prime importance with the equipment used, so long as the origins on all the strips were at the same side of the tank, as there was a current reversal switch so the anode/cathode positions could be fixed after the equipment was set up) and the restraining bars were put into position to keep some tension on the strips, whose ends were submerged in each buffer compartment. Current was passed through the strips for about 15min before application of the samples to allow equilibration.

Samples were applied using 20 μl microcaps. It was found that when microcaps were placed vertically in a solution the fluid that rose by capillary action alone had an average volume of 12 μl (by weight). As it is not necessary to fill the microcap completely using the rubber bulb (and of course risk contamination of the bulb with highly

radioactive solutions), '12 μ l' samples were routinely applied to the paper. These were expelled gradually along the marked origin using slight pressure in the rubber bulb and immediately after application of a sample to the electrophoresis strip, a similar sample (using the same microcap) was applied to a small strip (6cm x 0.5cm) of Whatman No1 paper in a Luckhams tube containing also 1ml distilled water and the strip then submerged in the water. This was assayed for radioactivity and used as a standard for the experiment. It was found that counting a paper strip submerged in 1ml of distilled water was more effective than counting the strip alone (see Table 2.4.).

The electrophoresis was run at 400v, 2mA for various times up to 1h. The papers were then removed and using the premarked pencil lines cut into 0.5cm strips (beginning at the origin end of the paper) which were then placed in labelled Luckhams tubes containing 1ml of distilled water. The paper strips were all submerged and then the tubes assayed for radioactivity. By running standards of known composition against unknowns and plotting radioactivity corrected for background, against strip number it was possible to determine the composition of unknown sample by comparing the position of the peaks obtained; measurement of the radioactivity in each peak and comparison with the total radioactivity in the sample applied gave an estimate of the percentage of each component.

TABLE 2.4. EFFICIENCY OF COUNTING METHODS FOR PAPER STRIPS

| Sample | Paper Strip | Paper Strip/1mlH ₂ O | 1mlH ₂ O |
|--------|-------------|---------------------------------|---------------------|
| A | 100.9% | 151.2% | 107.9% |
| B | 27.5% | 67.7% | 93.4% |
| C | 71.0% | 114.5% | 89.6% |
| D | 74.1% | 162.0% | 140.8% |
| MEAN | 68.4% | 123.9% | 107.9% |

1ml samples of the following were assayed for radioactivity:-

| | |
|---|--|
| A | [¹²⁵ I]iodide |
| B | ¹²⁵ I-PVP |
| C | } ¹²⁵ I-PVP with some [¹²⁵ I]iodide |
| D | |
| | contamination |

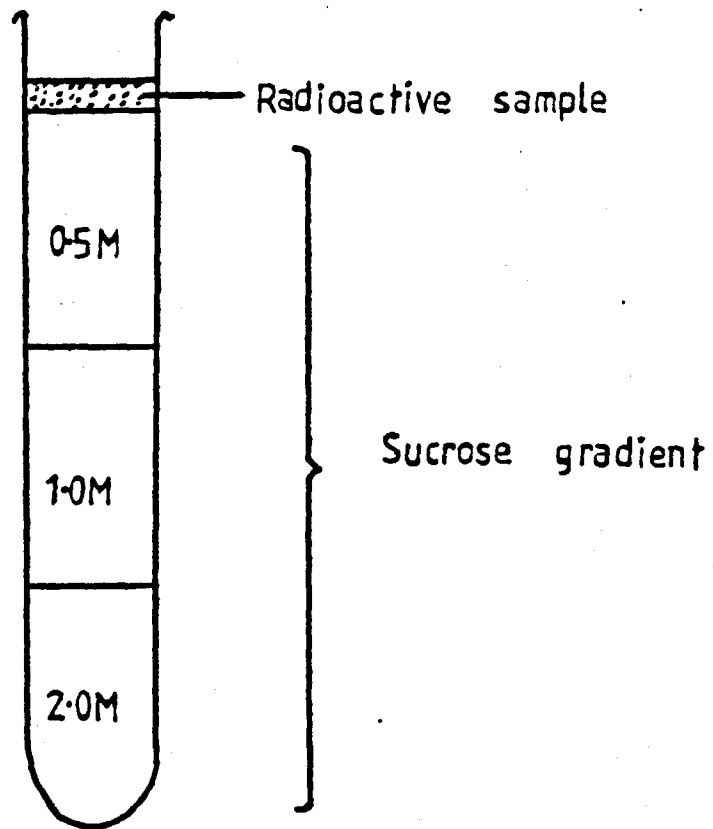
Then 12 μ l samples of A - D were assayed for radioactivity after application to a paper strip which was either used as it was or immersed in 1ml of distilled water.

The radioactivities recorded were expressed as a percentage recovery by relating them to the original standards.

2.9 DENSITY GRADIENT CENTRIFUGATION

Various aspects of differential centrifugation and density gradient separation are well documented by Reid (1971). Here a relatively crude discontinuous sucrose gradient was used to estimate the aggregation of colloidal ^{198}Au in the presence of various modifiers. Equal volumes (7ml) of 0.5, 1.0 and 2.0 M sucrose were gently pipetted into three polycarbonate centrifuge tubes (23ml), care being taken to prevent mixing of the layers (see Fig.2.4.) Samples (1ml) of colloidal ^{198}Au in medium 199 and 10% calf serum with or without modifier were carefully layered onto the surface of the gradient and then centrifugation was performed at 75,000g for varying times up to 2h in an MSE Superspeed 50 with a swing-out rotor of 3 x 23ml capacity. Centrifugation was carried out either at ambient temperature or 5° C. Samples of 1ml were then removed sequentially from the top of the gradient using a Finnpiptette and assayed for radioactivity. A duplicate of the sample applied was also assayed for radioactivity to allow estimation of the percentage recovery. From these data sedimentation profiles were prepared.

FIG. 2.4. DISCONTINUOUS SUCROSE DENSITY
GRADIENT



2.10 COLUMN CHROMATOGRAPHY

2.10.1. Sephadex Gel Filtration

Pharmacia Chemicals (1966) provide a useful handbook on gel filtration and the methodology was taken from here. Two types of Sephadex were used in the G-series and these were G-25 and G-10.

The Sephadex was normally supported in a distillation column 40-50cm long and an approximate internal diameter of 2.5cm and the bed height was usually approximately 30cm. The quantity of Sephadex that would be required was estimated and left overnight in a beaker containing excess solvent (a sodium acetate 0.02M, sodium azide 0.05% buffer was used) to allow swelling. Air bubbles trapped in a gel slurry can be a problem, and consequently may need to be removed by boiling the mixture or deaerating. This was not found to be a problem with the grades of Sephadex employed here so the column was packed immediately after swelling. The column was placed in a clamp stand and adjusted into a vertical position (this is very important to ensure even packing). The exit flow was sealed and the column one third filled with buffer solution (as above), then the gel slurry stirred and poured into the column. The exit flow was now opened and the column allowed to 'drip' into a suitable vessel. As the gel settled a head of clear solution appeared above it and this was drained until the fluid level almost reached the gel bed. Now the procedure was repeated, first stirring the top layer of the gel to stop a layering effect and then replenishing the supply of gel slurry. This was continued until the desired

bed volume was attained.

Using a peristaltic pump to maintain constant flow of adequate speed, buffer was run through the gel overnight to pack it down. As the columns were to be used for separation of PVP samples this buffer stock contained $40 \mu\text{g/ml}$ of PVP - 40 so that saturation of any non-specific binding sites for PVP would be achieved. The column was then ready for use, and continually kept moist to prevent cracking. If a column accidentally dried out and any cracks appeared it was repacked before use. Before application of samples most of the elutant above the surface of the gel was removed using a piece of rubber tubing attached to a 10ml syringe and the rest allowed to soak into the gel. Samples (usually 1ml) were gently dropped onto the surface of the gel using a syringe until a uniform covering was obtained. The sample was then allowed to soak into the surface of the gel before 5-10ml of buffer was carefully dropped onto it. This was subsequently removed by syringe and carefully replaced by an equivalent volume of buffer to minimize diffusion of the sample and produce sharper elutant peaks. The elutant was collected in 1-2ml aliquots using Luckhams tubes mounted in a fraction collector, each tube accumulating fluid for 2.5-5min. These tubes were then assayed for radioactivity and an elution profile prepared by plotting counts obtained against fraction number.

The void volume is the elution volume for a substance that is completely excluded from gel and is equivalent to the interstitial liquid between the gel beads and the position of the void volume on an elution profile can be measured using a coloured marker such as dextran blue or alternatively by measuring the elution of a

^{125}I -labelled compound which is known to be beyond the size range of the gel used. Here the position of the void volume was estimated from the elution patterns obtained for ^{125}I -labelled high molecular weight PVP known to be excluded by the gels used.

2.10.2 Anion-Exchange Chromatography.

Useful texts relating to methodology are provided by Pharmacia Fine Chemicals (1970) and BDH Chemicals Ltd. (1977). A zerolit (FF(ip)) anion exchange resin was used and this is a highly basic resin containing quaternary ammonium groups on a cross linked polystyrene matrix, supplied in the chloride form. Before filling the column the resin was fully hydrated with demineralised water. The column (again a general distillation column of approximately similar dimension to that used before) was partially filled with water and the resin slurry added, excess water being drained from the column. This was repeated until the column packed down and charged by running through deionised water overnight using a peristaltic pump to maintain constant flow. The resin was never allowed to dry out, as cracking occurs. This is of less importance when using an exchange resin, as separation is a charge-determined effect, but ideally an intact, bubble free column should be used.

Samples were applied as for sephadex gel columns and approximately 1ml fractions collected in a similar manner. From the elution profile it is possible to estimate the approximate time of appearance of the uncharged material and during subsequent runs this may be collected in a single container at the appropriate time. After a run the column must be regenerated

by displacing the bound ion and converting the resin back to its original form. In this case it was achieved by washing the column with hydrochloric acid (5% w/v), a volume of 350ml per 100ml of moist resin was used.

As the anion exchange resin was used to extract [^{125}I]iodide from ^{125}I -PVP preparations it was possible to detect the retention of radioactivity on the column after regeneration. This was found to be fairly considerable.

2.11. PREPARATION OF YOLK SAC FOR ELECTRON MICROSCOPY

Yolk sacs were prepared for electron microscopy according to the method of Pratten (1976) which was devised for the fixation of rabbit parotid gland. There were some slight modifications to the procedure and the overall scheme is outlined below.

Immediately after dissection from the mother or after a period of culture, pieces of yolk sac were fixed in 1.5% glutaraldehyde in cacodylate buffer for 3h.

| <u>Buffer Stock</u> | <u>Fixative</u> |
|---|---|
| 16.05g sodium cacodylate | 16ml buffer stock |
| 200ml distilled water brought to pH 7.3 with 1 N HCL and made up to 250ml | 3ml 2.5% TAAB glutaraldehyde 31ml distilled water. |

They were then buffer washed (equal parts buffer stock and distilled water), once for 20min and then twice for 15 min, and fixed in 1% OsO_4 (in cacodylate buffer stock). After dehydration in acetone the pieces of yolk sac were impregnated with Spurr's epoxy resin (1969) for 30min and then overnight in fresh resin. After 5h in fresh resin they were embedded in flat trays and the resin polymerized by heating to 60°C overnight. Pale gold to silver sections were cut (approximately 70nm in thickness) using a Reichert OmU2 ultramicrotome and these were mounted on copper grids and examined unstained using a Philips EM200 electron microscope (at 60Kv).

2.12. STATISTICAL ANALYSIS

Statistics forms an integral part of the description and interpretation of data obtained in all branches of Science. Although the data obtained here do not easily lend themselves to the comparisons made possible by the many tests for levels of significance that are available, other more general descriptive parameters are used throughout. The following texts were found particularly helpful Bailey (1959), Colquhoun (1971) and Elliott (1971).

2.12.1 Mean, Standard Deviation, Standard Error.

These values were routinely derived from the data, presented in this thesis so it is important to consider the underlying assumptions that are made in order to calculate such terms. All three are descriptive of a variable that follows a normal distribution, but with the small number of observations which were made in each experiment it is difficult if not impossible to confirm that the data obtained here fits any particular distribution. This difficulty is not uncommon and it is usually assumed that the normal distribution can be applied.

The arithmetic mean was used to describe the 'average' result obtained in any set of data.

Two quantities are frequently used to describe the variations between individual observations obtained in a series of replicate experiments and these are the standard deviation and standard error. It is important to understand the definitions of these terms and know where to apply them correctly. The standard deviation estimates the scatter of single observations from the population and 95.4% of the area under the normal distribution curve lies within the mean $\pm 2 \times$ standard deviation. It gives a measure of the inherent

variability within the population and is useful for describing the spread of the normal distribution of the observations made. The standard error (standard deviation of the mean) is more useful as a measure of accuracy of the mean, and it is defined as the estimated standard deviation of the normal distribution of the mean values of several samples taken from the population (as distinct from the individual sample values). Means and their standard errors should be used when comparing data derived from two different populations such as observations made under control and test conditions. As the majority of experimentation carried out in this thesis is not descriptive, but concerned with the evaluation of one set of data against another the observations were always described by a mean \pm standard error unless otherwise stated.

2.12.2. Correlation of measurements.

Routinely regression analysis was undertaken with uptake data that were approximately linear with time so as to calculate the best fit straight line, and hence obtain an estimate of the rate of uptake (Endocytic Index). The regression line has the following equation:-

$$y = a + bx$$

the constants can be calculated as follows:-

$$b = \frac{\sum (x - \bar{x}) (y - \bar{y})}{\sum (x - \bar{x})^2}$$

$$a = \bar{y} - \bar{b}x$$

In practice a simple computer programme was used to estimate the

regression coefficients.

It is only justifiable to calculate an Endocytic Index when uptake is linear with time, with an acceptable amount of scatter. What was definable as an acceptable amount of scatter was usually quite discernable by eye, but it was found useful to have a quantitative estimate of scatter about the estimated regression line. As the same computer package gave a correlation coefficient for the data this was usually used as an estimate of scatter. However, the type of data produced here is not ideally suited to definition by this coefficient. For this procedure the two variables (i.e. uptake and time of sampling in this case) should approximately follow a bivariate normal distribution, and it is obvious that the variables considered here do not - an important point which is often overlooked. The following quotation is taken from Bailey (1974):-

"a popular index of the degree of association between continuous variables is the correlation coefficient. This is often applied in a rather loose and uncritical way to any data involving double classification of the type under discussion. Although such methods may be quite useful at times, they are not recommended. The reason is that the interpretation and distribution of the correlation coefficient are clearly understood only in certain rather special circumstances and unless these are approximately fulfilled there may be considerable doubt about the statistical significance of the results obtained".

When applied correctly correlation coefficients assume values between -1 and +1, -1 being a perfect negative correlation and +1 being a perfect positive correlation. By calculating a value for the

type of uptake data described here, the coefficients are unrealistically high and do not really relate to the absolute scale. However, the correlation coefficients can still be of some use as an internal comparison between experiments rather than as universal absolute values, and were used only in this context.

$$\text{Correlation Coefficient } r = \frac{\sum (x - \bar{x}) (y - \bar{y})}{\sqrt{\sum (x - \bar{x})^2 \sum (y - \bar{y})^2}}$$

Perhaps a more meaningful indication of the reliability of the regression coefficient would be the standard error of the regression coefficient, which was also calculated but is not quoted. This also has its problems as it is most usefully estimated when n is large (i.e. > 30) and here n was usually 10

$$\begin{aligned} \text{Standard Error of regression Coefficient} &= \frac{S}{\sqrt{\sum (x - \bar{x})^2}} \\ \text{where } S^2 &= \frac{1}{n - 2} \left\{ \sum (y - \bar{y})^2 - \frac{[\sum (x - \bar{x}) (y - \bar{y})]^2}{\sum (x - \bar{x})^2} \right\} \end{aligned}$$

2.13. WASHING PROCEDURE

Although the general washing up procedure is of little relevance and probably corresponds to that employed in other laboratories the efficient cleaning of culture equipment was of vital importance to the whole culture technique. All adherent radioactivity had to be displaced from the culture flasks before re-use and this had to be achieved without the use of potentially toxic detergents. These factors were overcome by using the following method.

General washing Procedure

- a) Soak overnight in dilute detergent (pyroneg) solution
- b) Rinse in tap water, soak in dilute hydrochloric acid for 3-6h.
- c) Rinse in tap water and final rinse in distilled water.
- d) Place in drying oven at approximately 40°C

Culture Equipment Washing Procedure

- a) Sonicate for 1-2h in a 0.5 - 1.0% sodium carbonate solution.
- b) Rinse in tap water, soak for 1 - 3h in dilute hydrochloric acid.
- c) Rinse in tap water and final rinse in distilled water.
- d) Place in sterilizing oven at approximately 200°C.

Silicone rubber bungs were washed in the same way but sterilized by boiling.

CHAPTER 3.

EVALUATION OF ^{125}I -PVP, ^{125}I -dBSA AND COLLOIDAL ^{198}Au
AS SUITABLE PINOCYTIC MARKERS.

3.1. INTRODUCTION.

To investigate the pharmacology of pinocytosis it is essential to use a reliable method for quantitation of uptake. Until recently such techniques were not available so the collection of meaningful data was severely hampered. The concept of Endocytic Index as developed by Williams et al., (1975 a,b) now provides an effective tool for the quantitation of pinocytosis and the application of this procedure to the rat visceral yolk sac system, a very useful model system for studying pinocytosis, has allowed the detailed study reported here relating to the pharmacology of pinocytosis in this tissue. As such a large portion of the work reported in this thesis involves the potential modification of pinocytic uptake of radiolabelled substrates, it is important that the daily variability in Endocytic Index for each substrate is fully understood. The 'control' Endocytic Indices reported here provide an important background for the subsequent Chapters.

Any method for the quantitation of pinocytosis relies upon the use of dependable pinocytic markers, and here ^{125}I -PVP, ^{125}I -dBSA and colloidal ^{198}Au were routinely used as probes. The kinetics of uptake of these substrates has already been discussed (Section 1.3.2.) and some of the essential requirements for a pinocytic marker have also been mentioned (Section 1.2.). The most relevant points concerning the use of the three named radiolabelled substrates in the rat yolk sac system are as follows:-

1. Ideally the daily and inter-batch variation in the rate of substrate capture should be minimal.

2. ^{125}I -labelled compounds should be completely stable both during storage and culture. Adequate compensation can be

made for intracellular degradation of the substrate during culture, provided the ^{125}I -label remains bound to the degradation products. Progressive release of iodide would be highly undesirable.

3. The fate of the radiolabelled marker must be fully understood - do the markers monitor pinocytosis?

In this Chapter (1) and (2) above are investigated and (3) is discussed.

3.2. RESULTS.

3.2.1. Uptake of ^{125}I -PVP, ^{125}I -dBSA and colloidal ^{198}Au .

Table 3.1. shows the uptake of eighteen batches of ^{125}I -PVP, Table 3.2. the uptake of three batches of ^{125}I -dBSA and Table 3.3. the uptake of eight batches of colloidal ^{198}Au . Figs. 3.1. and 3.2. show typical experiments which describe the uptake of ^{125}I -PVP and colloidal ^{198}Au , and ^{125}I -dBSA respectively.

3.2.2. Effect of Storage time on the Percentage ^{125}I Iodide in a Batch of ^{125}I -PVP.

Fig. 3.3. shows a typical separation profile observed when a ^{125}I -PVP preparation containing a small percentage of ^{125}I iodide was subjected to electrophoresis for 35 min. From these results (corrected for background) the percentage ^{125}I iodide in the preparation was estimated either by summing the total activity on the paper to obtain a value for the total radioactivity in the sample applied or by counting a standard of equal volume to that applied to the electrophoresis paper. Table 3.4. shows the percentage free ^{125}I iodide measured in two different samples of a single batch of ^{125}I -PVP (171 BA). One portion was left in the vial supplied by the Radiochemical Centre, Amersham, which contained a sachet of anion exchange resin, the second was removed from this container at the beginning of the experiment and kept in isolation from any anion-exchange resin. At various time intervals two 12 μl aliquots were taken from both samples, one counted as a standard and the other subjected to electrophoresis. It can be seen that there is a very slow but progressive loss of ^{125}I iodide from the ^{125}I -PVP complex, but the conditions of storage did not affect the rate of release.

TABLE 3.1. UPTAKE OF DIFFERENT BATCHES OF ^{125}I -PVP SUPPLIED
BY THE RADIOCHEMICAL CENTRE, AMERSHAM.

| Date | Batch | Endocytic Index | Correlation Coefficient | Mean Endocytic Index (\pm S.E.) |
|----------|--------|-----------------|-------------------------|------------------------------------|
| 9/10/75 | 129 BA | 1.44 | 0.973 | 1.43 \pm 0.03 |
| 14/10/75 | " | 1.49 | 0.963 | |
| | " | 1.37 | 0.989 | |
| 29/10/75 | 130 BA | 1.09 | 0.985 | 1.20 \pm 0.04 |
| 11/11/75 | " | 1.39 | 0.932 | |
| 13/11/75 | " | 1.08 | 0.976 | |
| | " | 1.19 | 0.975 | |
| 18/11/75 | " | 1.17 | 0.901 | |
| 21/11/75 | " | 1.34 | 0.952 | |
| 28/11/75 | " | 1.17 | 0.965 | 1.86 \pm 0.33 |
| 10/12/75 | 132 BA | 2.48 | 0.870 | |
| 18/12/75 | " | 1.34 | 0.987 | |
| 30/12/75 | " | 1.76 | 0.994 | 1.45 \pm 0.12 |
| 29/4/76 | 136 BA | 1.34 | 0.983 | |
| 21/4/76 | " | 1.67 | 0.988 | |
| 15/6/76 | " | 1.29 | 0.979 | 1.94 |
| 6/9/76 | 140 BA | 1.94 | 0.982 | |

TABLE 3.1.(ii)

| Date | Batch | Endocytic Index | Correlation Coefficient | Mean Endocytic Index (\pm S.E.) |
|----------|--------|-----------------|-------------------------|------------------------------------|
| 14/9/76 | 142 BA | 2.39 | 0.997 | 2.25 ± 0.08 |
| 20/9/76 | " | 1.96 | 0.973 | |
| 29/9/76 | " | 2.35 | 0.970 | |
| 6/10/76 | " | 2.19 | 0.984 | |
| 11/10/76 | " | 2.37 | 0.977 | |
| 18/10/76 | 145 BA | 2.13 | 0.975 | 2.39 ± 0.34 |
| 20/10/76 | " | 3.06 | 0.989 | |
| 29/10/76 | " | 1.98 | 0.981 | |
| 20/12/76 | 146 BA | 2.29 | 0.992 | 2.44 ± 0.11 |
| 6/1/77 | " | 2.66 | 0.902 | |
| 17/1/77 | " | 2.37 | 0.989 | |
| 7/2/77 | 149 BA | 1.64 | 0.980 | 1.89 |
| 16/2/77 | " | 2.13 | 0.974 | |
| 3/5/77 | 150BA | 1.47 | 0.793 | 1.64 ± 0.10 |
| 11/5/77 | " | 1.83 | 0.964 | |
| 16/5/77 | " | 1.62 | 0.920 | |
| 2/6/77 | 153 BA | 1.82 | 0.966 | 1.64 ± 0.10 |
| 26/6/77 | " | 1.59 | 0.960 | |
| 20/7/77 | " | 1.51 | 0.962 | |

Table 3.1.(iii)

| Date | Batch | Endocytic Index | Correlation Coefficient | Mean Endocytic Index (\pm S.E.) |
|----------|--------|-----------------|-------------------------|------------------------------------|
| 29/8/77 | 156 BA | 2.80 | 0.989 | 2.25 \pm 0.32 |
| 31/8/77 | " | 1.71 | 0.956 | |
| 14/9/77 | " | 2.24 | 0.984 | |
| 5/10/77 | 158 BA | 1.62 | 0.992 | 2.06 \pm 0.22 |
| 10/10/77 | " | 2.29 | 0.961 | |
| 11/1/78 | " | 2.28 | 0.990 | |
| 18/1/78 | 161 BA | 1.71 | 0.607 | 2.23 \pm 0.19 |
| 23/1/78 | " | 2.60 | 0.977 | |
| 30/1/78 | " | 2.16 | 0.969 | |
| 6/2/78 | " | 3.19 | 0.984 | |
| 13/2/78 | " | 2.31 | 0.932 | |
| 23/2/78 | " | 2.54 | 0.981 | |
| 27/2/78 | " | 1.67 | 0.824 | |
| 3/3/78 | " | 1.67 | 0.983 | |
| 17/3/78 | 164 BA | 2.11 | 0.898 | 2.27 \pm 0.23 |
| 31/3/78 | " | 2.71 | 0.916 | |
| 15/4/78 | " | 1.98 | 0.979 | |

Table 3.1.(iv)

| Date | Batch | Endocytic Index | Correlation Coefficient | Mean Endocytic Index (\pm S.E.) |
|---------|--------|-----------------|-------------------------|------------------------------------|
| 20/4/78 | 166 BA | 2.63 | 0.855 | |
| 26/4/78 | " | 2.19 | 0.919 | |
| 30/4/78 | " | 2.12 | 0.966 | |
| 17/5/78 | " | 2.04 | 0.960 | 2.06 \pm 0.12 |
| 22/5/78 | " | 1.60 | 0.988 | |
| 12/6/78 | " | 1.86 | 0.951 | |
| 19/6/78 | " | 1.98 | 0.973 | |
| 10/8/78 | 169 BA | 2.10 | 0.976 | 2.10 |
| 8/9/78 | 171 BA | 1.93 | 0.931 | 1.93 |

Overall Mean

1.94 \pm 0.06 (62)

TABLE 3.2. UPTAKE OF DIFFERENT BATCHES OF ^{125}I -LABELLED
BOVINE SERUM ALBUMIN.

| Date | Batch | Endocytic Index | Correlation Coefficient | Mean Endocytic Index(\pm S.E.) |
|---------|-------|--------------------|----------------------------|--------------------------------------|
| 4/5/76 | VI | 57.07 | 0.933 | |
| 17/5/76 | | 43.29 | 0.995 | 51.70 \pm 4.26 |
| 4/6/76 | | 54.75 | 0.965 | |
| 11/2/77 | VIII | 82.04 | 0.978 | |
| 21/2/77 | | 108.74 | 0.921 | 78.03 \pm 13.31 |
| 28/2/77 | | 43.90 | 0.918 | |
| 7/3/77 | | 77.47 | 0.987 | |
| 23/5/77 | XI | 18.06 | 0.988 | 16.75 (2) |
| 20/6/77 | | 15.44 | 0.995 | |

TABLE 3.3. UPTAKE OF DIFFERENT BATCHES OF COLLOIDAL ^{198}Au

SUPPLIED BY THE RADIOCHEMICAL CENTRE, AMERSHAM.

| Batch | Approximate Date | Endocytic Index | Mean Endocytic Index (\pm S.E.) |
|--------------|------------------|--|------------------------------------|
| Batch 7 | March, 1976 | 3.84 | 3.84 |
| Batch 8 | May, 1976 | 4.41 1.60 | 3.00 |
| Batch 9 | August, 1976 | 2.54 3.08 | 2.81 |
| Batch 10 | November, 1976 | 3.70 2.86 3.44 2.65 2.52 | 3.03 ± 0.23 |
| Batch 11 | March, 1977 | 5.54 3.93 4.65 3.27 3.65 3.33 | 4.06 ± 0.36 |
| Batch 12 | August, 1977 | 3.11 2.95 4.88 2.38 | 3.33 ± 0.54 |
| Batch 13 | March, 1978 | 3.62 | 3.62 |
| Batch 14 | October, 1978 | 1.60 5.35 2.20 3.43 4.34 | 3.38 ± 0.69 |
| OVERALL MEAN | | | 3.42 ± 0.20 |

FIG. 3.1. TYPICAL EXPERIMENTS TO SHOW THE
UPTAKE OF ^{125}I -PVP & COLLOIDAL ^{198}Au

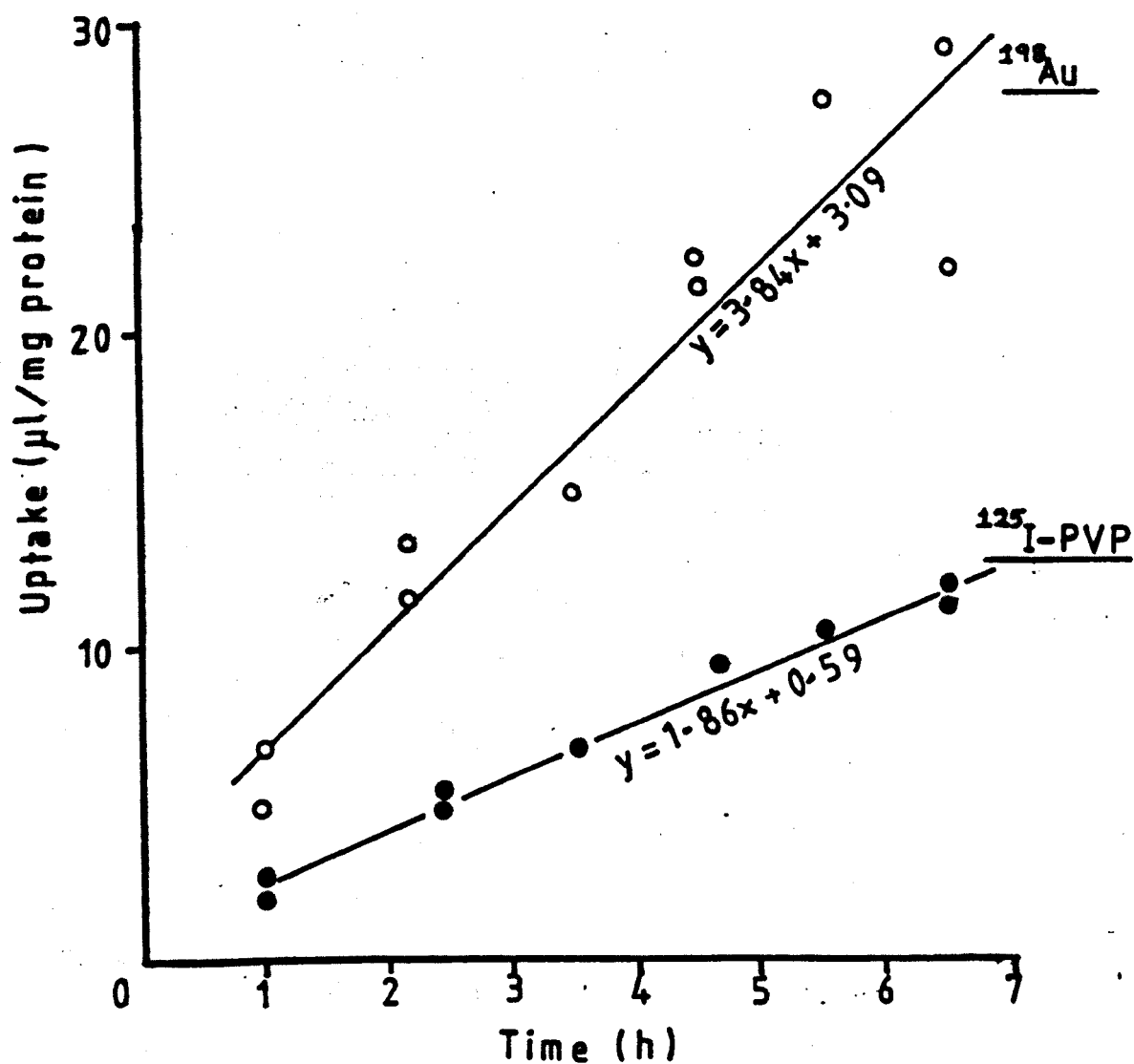


FIG. 3.2. A TYPICAL EXPERIMENT TO SHOW
THE UPTAKE OF ^{125}I -dBSA

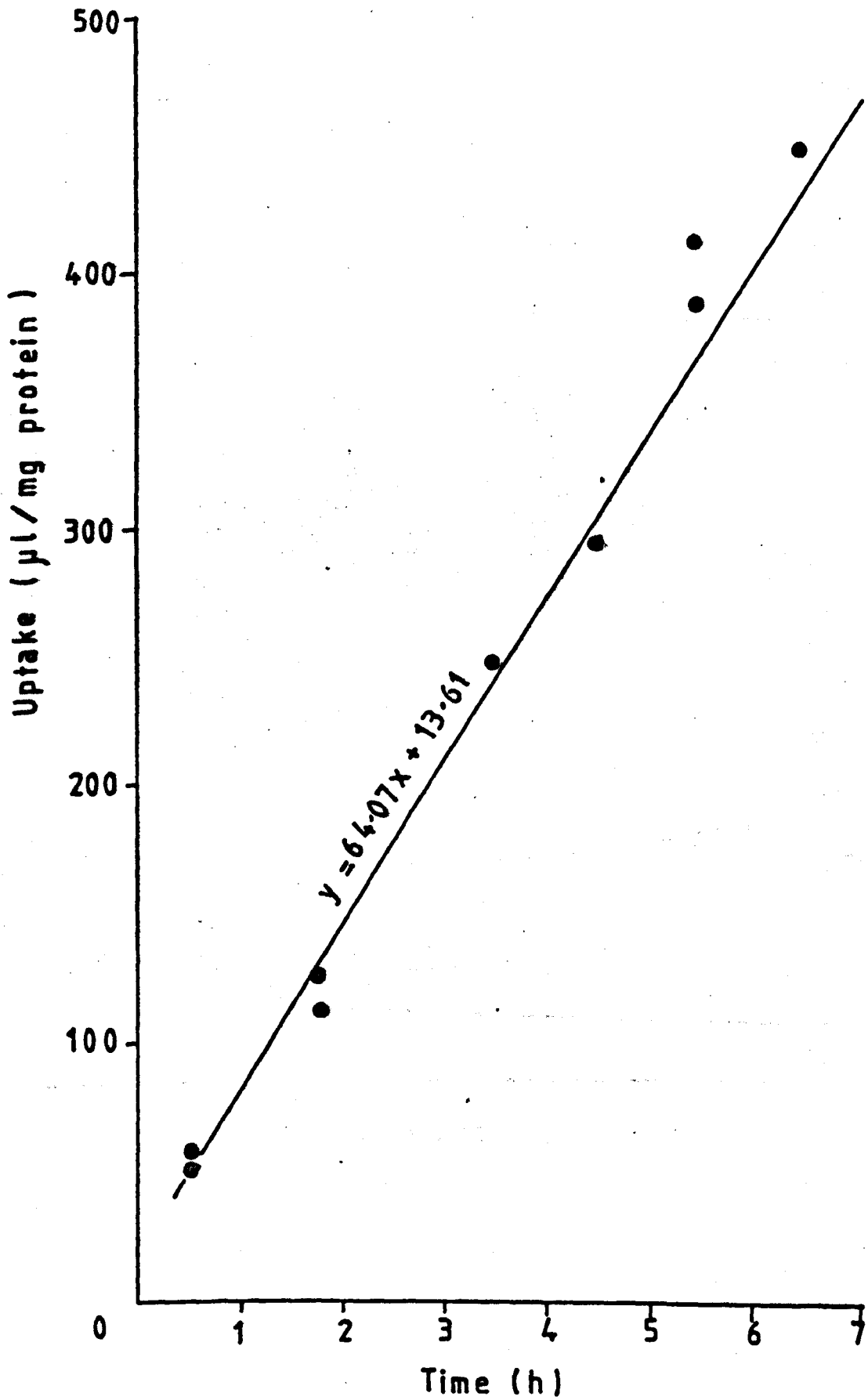


FIG.3.3. ELECTROPHORESIS OF AMERSHAM ^{125}I -PVP

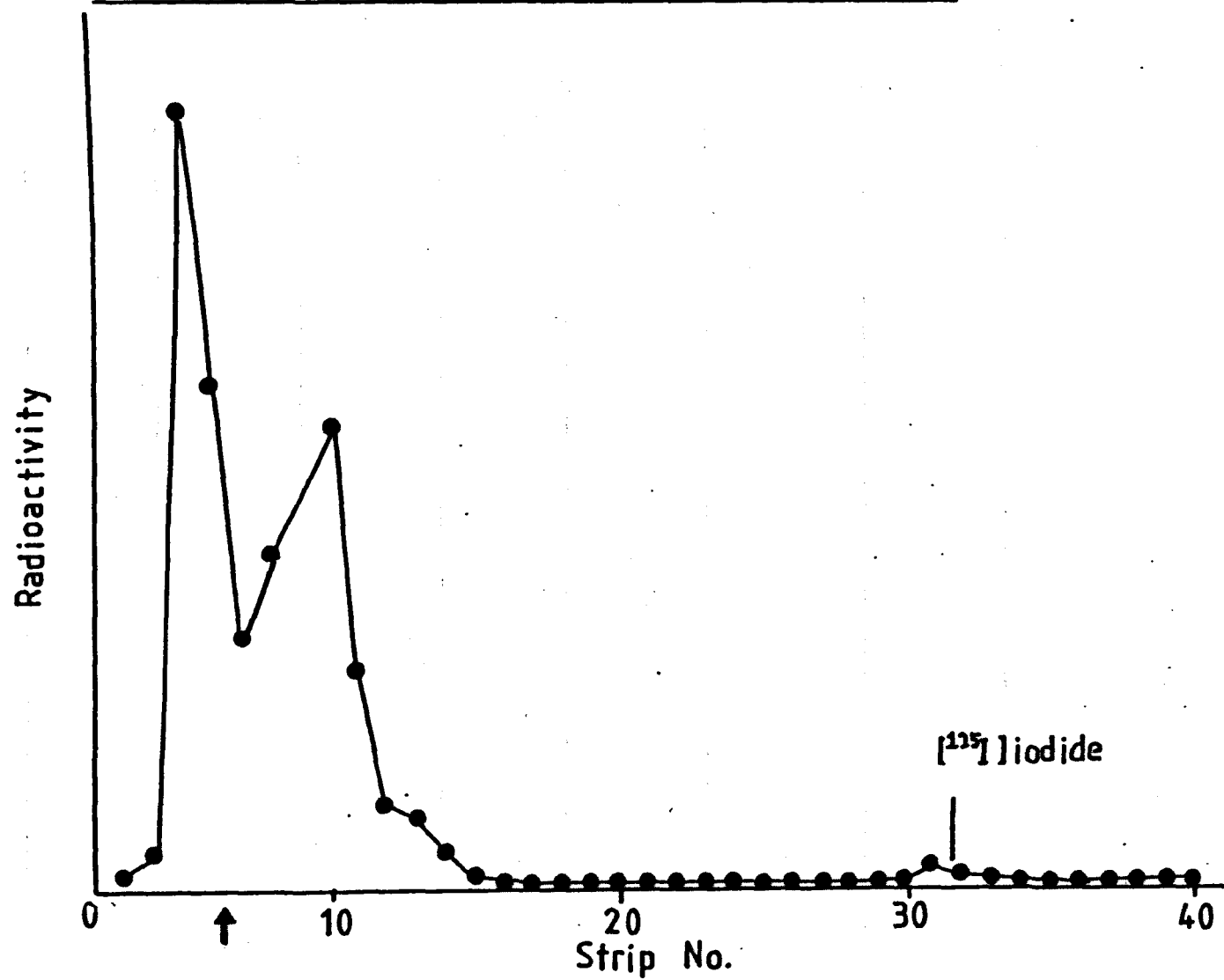


TABLE 3.4. EFFECT OF STORAGE TIME ON PERCENTAGE $[^{125}\text{I}]$ IODIDE
IN A PREPARATION OF ^{125}I -PVP.

| Days after Separation | Percentage of $[^{125}\text{I}]$ Iodide | |
|--------------------------|---|------------------------|
| | Stored with Anion Exchange Resin | Stored in Isolation |
| 2 | 0.36% | 0.42% |
| 6 | 0.34% | 0.55% |
| 13 | 0.58% | 0.67% |
| 20 | 0.71% | 0.79% |
| 27 | 0.52% | 0.74% |
| 34 | 0.53% | 1.20% |
| 41 | 1.91% | 2.04% |
| 48 | 0.67% | 1.49% |
| 55 | 0.69% | 1.68% |

3.2.3. Effect of incubation in culture medium on the Percentage

$[^{125}\text{I}]$ Iodide measured in a Preparation of ^{125}I -PVP.

Table 3.5. shows the percentage free $[^{125}\text{I}]$ iodide measured in samples of ^{125}I -PVP (Batch 172 BA) placed in medium 199 (containing 10% calf serum) and cultured for 6.5h with or without a rat yolk sac. Culturing did not cause any appreciable increase in the percentage $[^{125}\text{I}]$ iodide detectable within the ^{125}I -PVP preparation.

TABLE 3.5. EFFECT OF INCUBATION ON THE PERCENTAGE $[^{125}\text{I}]$ IODIDE
IN PREPARATION OF ^{125}I -PVP

| Replicate | Percentage $[^{125}\text{I}]$ iodide | | |
|-----------------|--------------------------------------|------------------|---|
| | Initially | Without yolk sac | Post Incubation (5.5h) With yolk sac |
| 1 | 1.06% | 2.45% | 2.20% |
| 2. | 1.09% | 1.99% | 2.20% |
| 3 | 0.95% | 2.36% | 0.94% |
| <hr/> | | | |
| Mean \pm S.E. | 1.03 \pm 0.04% | 2.27 \pm 0.14% | 1.78 \pm 0.42% |

3.3. DISCUSSION.

3.3.1. ^{125}I -PVP

PVP is a linear polymer which is believed to have a coiled secondary structure (Fig. 3.4). In water solution, clusters of water molecules are held to the polymer especially in the vicinity of the polar nitrogen and carbonyl oxygen atoms. It is probable that most PVP samples contain some branched chains, and the end groups are uncertain. The average molecular weight of the compound supplied by the Radiochemical Centre, Amersham is 33,000, but the weight distribution covers a range from less than 10,000 to greater than 80,000 (Fig. 3.5). The way in which iodide is bound to the molecule is still something of a mystery and actual methods of radiolabelling are discussed later (See Chapter 9).

The content of free ^{125}I iodide and subsequent stability of the product supplied by the Radiochemical Centre was investigated. In the specifications supplied with this compound it is stated that the free ^{125}I iodide content is always less than 5%. At the outset of the investigation the content of free ^{125}I iodide in batch 171BA of ^{125}I -PVP was 0.36% but it should be noted that this was several weeks after the shipment was supplied to the laboratory. The inclusion of an anion exchange sachet in the dispensing vial made one suspect that there may be continual loss of ^{125}I iodide from the polymer complex, the resin perhaps acting to maintain a low percentage of free iodide in the preparation. If this were the case an increase in the percentage free ^{125}I iodide would not be detectable until the exchange resin became saturated and for this reason a portion of ^{125}I -PVP was isolated from the stock solution.

FIG. 3.4. STRUCTURE OF PVP

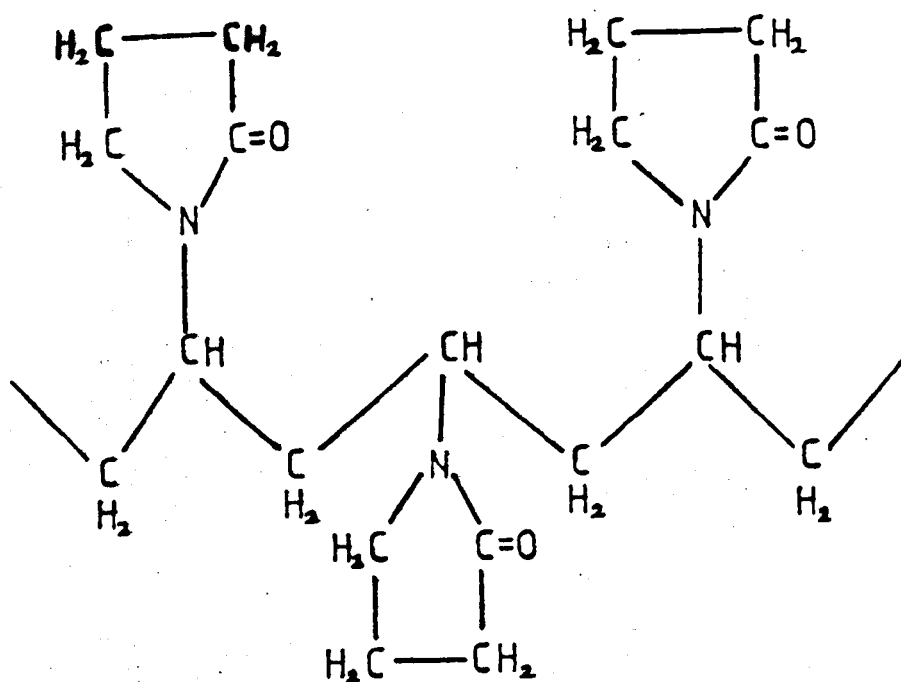
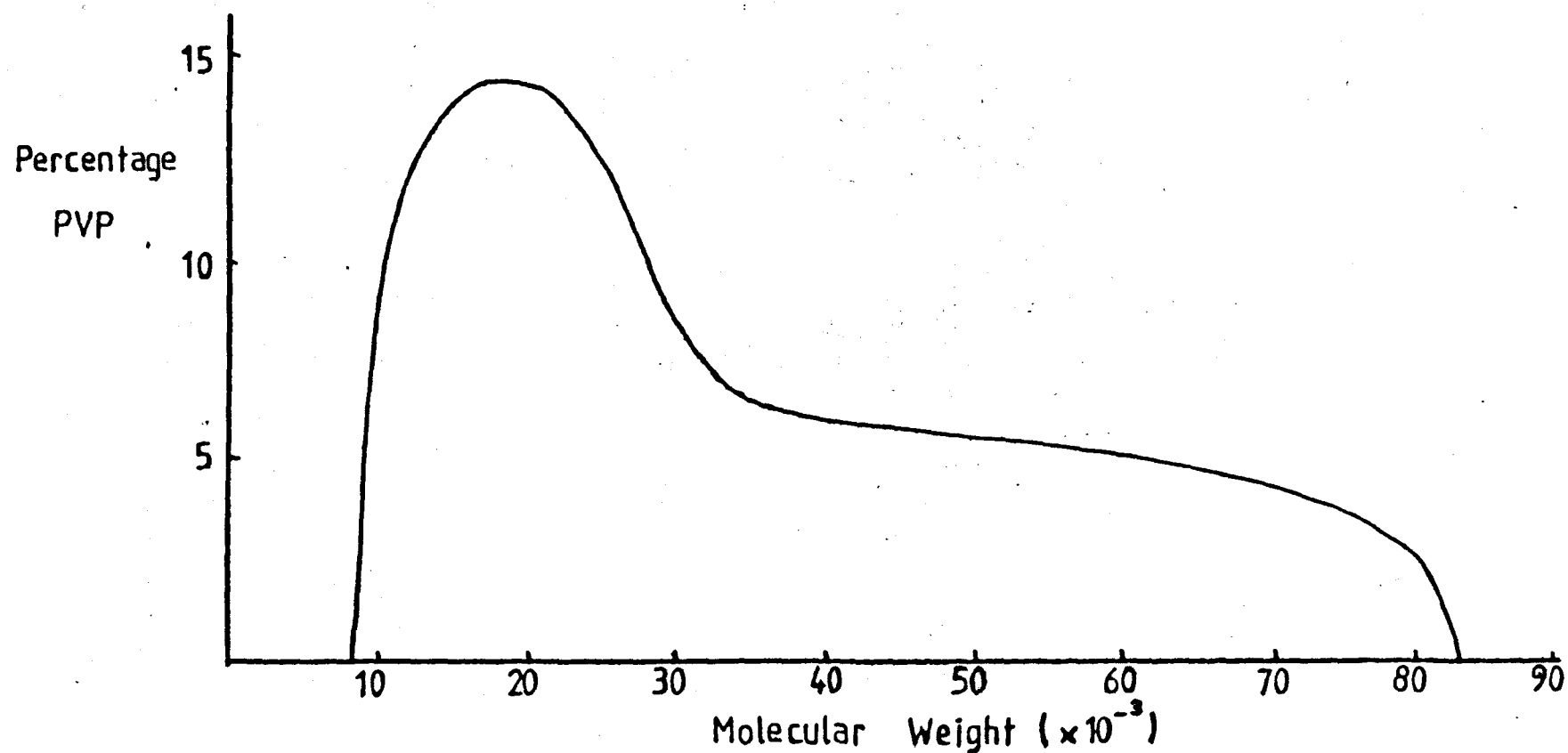


FIG. 3.5. MOLECULAR WEIGHT DISTRIBUTION FOR ^{126}I -PVP

(Supplied by the Radiochemical Centre, Amersham)



The free [^{125}I] iodide content of both the isolated portion and normal batch of ^{125}I -PVP were monitored by paper electrophoresis over a period of seventy days. During this time the percentage [^{125}I] iodide rose from 0.36% to 0.69% in the vial containing ^{125}I -PVP and the exchange resin, and likewise rose from 0.42% to 1.68% without resin. The percentage [^{125}I] iodide after 41 days of storage was 1.91% (+ resin) and 2.04% (- resin) and these values were slightly higher than those observed at the other time intervals, and did not fit into the overall increasing pattern. (It is not felt that these are particularly significant results, their greater magnitude possibly being due to some experimental error). Although the [^{125}I] iodide content of batch 171BA of ^{125}I -PVP did increase marginally with age, removal of the anion exchange resin did not produce a dramatic increase in the appearance of [^{125}I] iodide; in fact the value observed after 70 days was only some twofold greater than with the resin sachet present. In any case the percentage of [^{125}I] iodide detectable at all stages was well within the specifications of the manufacturer, and so the ^{125}I -PVP supplied was certainly suitable for the uptake studies undertaken here.

The [^{125}I] iodide content of a typical preparation of ^{125}I -PVP may be low on removal from the storage vial but it was considered important to take this investigation one step further and look at the effect of incubation in medium 199 and calf serum on ^{125}I -PVP stability. These experiments were carried out with batch 172BA and the data in Table 3.5. show that incubation of ^{125}I -PVP for approximately 6h at 37°C with or without a yolk sac resulted in a

twofold increase in free $[^{125}\text{I}]$ iodide content. Before incubation the preparation contained 1.03% $[^{125}\text{I}]$ iodide (a higher initial level than observed for batch 171BA), whereas following incubation either with or without a yolk sac the equivalent percentages were 1.78% and 2.27% respectively. These changes were probably trivial and would not greatly affect the observed uptake pattern attributed to the capture of ^{125}I -PVP. Free $[^{125}\text{I}]$ iodide in the culture medium would rapidly diffuse into the yolk sac cells until an equilibrium was reached and this process would easily be complete before the first sample usually taken at 1h. A portion of the radioactivity measured within the tissue would thus represent $[^{125}\text{I}]$ iodide and not ^{125}I -PVP, but this quantity would remain constant throughout the experiment and only affect the intercept of the estimated regression line and not its gradient i.e. the Endocytic Index calculated would relate directly to the uptake of ^{125}I -PVP. Problems arise if the $[^{125}\text{I}]$ iodide content rises appreciably as the medium radioactivity is used to estimate the uptake of ^{125}I -PVP. If a large portion of the medium counts represent $[^{125}\text{I}]$ iodide the uptake of ^{125}I -PVP will be underestimated.

From Table 3.1. it can be seen that the rate of uptake of ^{125}I -PVP measured for eighteen batches of ^{125}I -PVP show remarkable uniformity, the overall mean value being $1.94 \pm 0.06 \mu\text{l/mg protein/h}$ calculated from 62 individual Endocytic Indices. This mean value is very similar to that observed for ^{125}I -PVP by several users of this substrate (Williams *et al.*, 1975,a; Ibbotson 1978, Livesey 1979). There was a fairly wide range in Endocytic Indices observed, the lowest being 1.08 and the highest being $3.19 \mu\text{l/mg protein/h}$ and throughout the three years of experimentation there did appear

to be some variability in the magnitude of the mean Endocytic Indices observed for batches of ^{125}I -PVP. It was very difficult to pinpoint the factors governing this fluctuation. Perhaps there was true batch variation in the properties of the ^{125}I -PVP supplied, alternatively other consumables such as medium 199, calf serum and O_2/CO_2 gas cylinders were also subject to daily variation and also likely to influence experimental results. It was impossible to correlate the changes in Endocytic Index with any of these variables. Of course, it was quite possible that the biological system under investigation was susceptible to variation. Rats, and hence the physiology of the yolk sac, may respond differently to variations in diet, routine, seasonal changes etc., and, although steps were taken to minimize these effects, some were beyond control. The information reported here concerning ^{125}I -PVP supplied by the Radiochemical Centre, Amersham is of particular interest when compared with the data shown in Chapter 8. There the properties of ^{125}I -PVP prepared in the laboratory are described and discussed.

3.3.2. ^{125}I -dBSA

Bovine serum albumin (BSA) and human serum albumin (HSA) have been used quite extensively as probes to study pinocytotic uptake and intracellular degradation (Williams et al., 1975 b;; Buys et al., 1973; Moore et al., 1974; Ricketts & Rappitt, 1975; Ehrenreich & Cohn, 1967; Nilsson & Berg, 1977; Ryser & Hancock, 1965; Brownstone & Chapman - Andresen, 1971). Its size (molecular weight 65 - 70,000) makes it fairly representative of proteins as a whole and, as many conformational studies have been undertaken, its structure is at least partially understood. BSA was chosen as a degradable substrate

for the comparative studies undertaken in this thesis as it was already under investigation within the laboratory, the results of these experiments being extensively discussed by Moore (1975), Ibbotson (1978), and Livesey (1979). The rate of capture of albumin is known to vary according to conformational changes that can be induced by chemical treatment (Moore et al., 1974, 1977). Here a formaldehyde-treated protein was used and the Endocytic Indices obtained for three different batches are shown in Table 3.2. Considerable variation can be seen, the mean values being 51.70 ± 4.26 , 78.03 ± 13.31 and $16.75 \mu\text{l/mg protein/h}$. Batch XI was not used extensively as the rate of uptake was so low, it was felt that the formaldehyde-denaturation had not been successfully completed. The other two mean values correspond well to those reported by Moore and Ibbotson for similar preparations. It is apparent that the Endocytic Indices calculated for the protein showed slightly more variability than those reported for $^{125}\text{I-PVP}$. The standard error of the mean for the overall mean $^{125}\text{I-PVP}$ value was only 3% of the total value, whereas the two equivalent percentages that can be calculated for batches of $^{125}\text{I-BSA}$ are 8% and 17% respectively. However, the batch of $^{125}\text{I-PVP}$ that showed the most variation had a standard error that was 14% of the mean, so the $^{125}\text{I-PVP}$ Endocytic Indices are not vastly more reliable than those measured for $^{125}\text{I-dBSA}$, but only seem so perhaps because the figures obtained are somewhat smaller. $^{125}\text{I-dBSA}$ does show a significant batch variation probably due to undetectable inconsistencies in preparation resulting in varying degrees of denaturation. As far as possible experiments designed to detect any effect of potential modifiers were carried out with a single batch of $^{125}\text{I-dBSA}$, but

in any case a matched control experiment was performed with each test experiment so that direct comparison of effects could be achieved even if more than one batch had to be used.

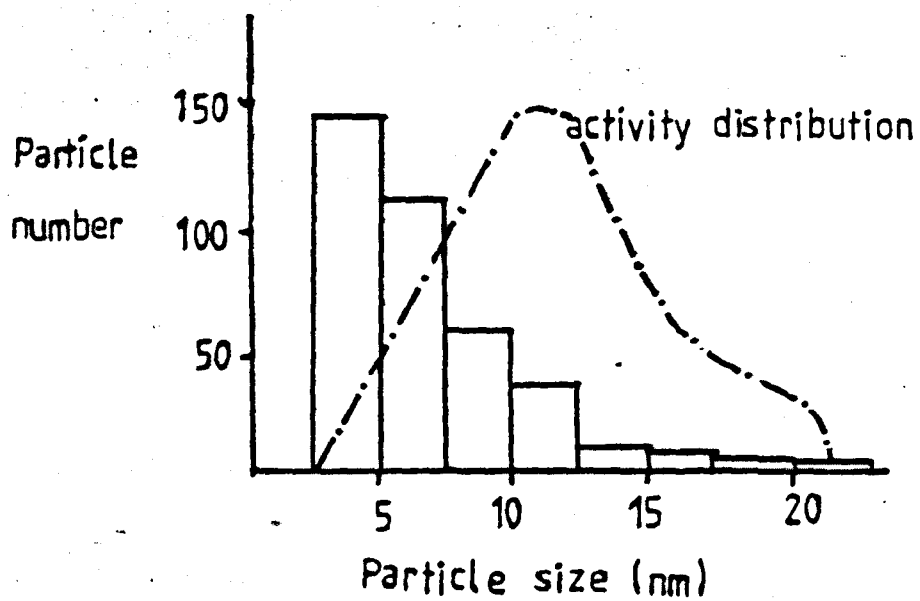
The problems of substrate stability that were discussed at length for ^{125}I -PVP also apply to ^{125}I -dBSA. During the course of an incubation the percentage TCA soluble material increases from a base-level which is normally less than 2%. Although the initial activity may be attributable to a low concentration of free ^{125}I iodide the progressive increase has been attributed to the degradation of ^{125}I -dBSA to produce fragments of ^{125}I -tyrosine and glycyl- ^{125}I -tyrosine which were identified by (Williams et al., 1975 b). ^{125}I -dBSA appears to be a reliable and very useful degradable substrate.

3.3.3. Colloidal ^{198}Au .

The colloidal ^{198}Au used was a suspension of metallic ^{198}Au gold stabilized with gelatin (20 mg/ml) and glucose (200 mg/ml). The particle size was variable, the maximum size being 20nm, and about 80% of the radioactivity present in particles between 5 and 20nm (Fig. 3.6. shows the size and radioactivity distributions). Colloidal ^{198}Au was not an easy isotope to handle as it has a short half life (65h) and exhibits high energy emission, but it still provided an interesting addition to ^{125}I -PVP and ^{125}I -dBSA. Earlier studies had shown some interbatch variation in the Endocytic Index for colloidal ^{198}Au (Roberts et al., 1977), but the data reported here display little interbatch variation, the overall mean Endocytic Index being $3.42 \pm 0.20 \mu\text{l/mg protein/h}$ (See Table 3.3). The values shown here overlap with the last batch described by Roberts et al., (1977) which they found to have a mean Endocytic Index of $3.22 \mu\text{l/mg protein/h}$

FIG. 3.6. PARTICLE SIZE AND ACTIVITY
DISTRIBUTIONS FOR COLLOIDAL ^{198}Au
(GCS 1P)

(Supplied by the Radiochemical Centre, Amersham)



and the present data relate to subsequent batches from the same source. Some intrabatch variation was also observed, most markedly by batch 14 which displays a range from 1.06 to 5.35 $\mu\text{l}/\text{mg protein/h}$. The overall standard error was only 6% of the mean, so the Endocytic Indices showed an acceptable degree of reliability, albeit less than observed for ^{125}I -PVP.

3.3.4. Fate of Radiolabelled Markers.

A criticism levelled at many experiments involving the use of radiotracers to quantitate pinocytosis is that the fate of the marker is always assumed to be entirely a directional transport via pinosomes to the lysosomal compartment of the cell. If the radiotracer has one or more different fates, the extrapolation from cell or tissue radioactivity to pinocytic rate becomes difficult at best and at worst totally meaningless. The following are just some of the possibilities that may be envisaged; the existence of one or more of these would lead to inaccuracy:-

1. Binding of radiolabelled substrate to the exterior of the cell, either rapid or progressive, particularly if this is not followed by internalization.
2. Penetration of radiolabelled substrate into intercellular spaces where it is inaccessible to the washing procedure.
3. Uptake of radiolabelled substrate into the cell by some mechanism other than pinocytosis; diffusion, active or passive transport etc.
4. Rapid release of radiolabelled substrate by an exocytic mechanism.

These points are now considered in relation to the radiotracer

technique employed here to quantitate pinocytosis in the rat visceral yolk sac. The final possibility (4) has already been discussed and rate of exocytosis of the substrates employed here were all found to be relatively small (less than 5%/h) (Williams et al., 1975 a,b; Roberts et al., 1977), so this is one factor that need not be feared in this system. The first three points, if operational, would all lead to an overestimation of pinocytic uptake. It is highly unlikely that macromolecules such as ^{125}I -PVP and ^{125}I -dBSA, and also colloidal ^{198}Au would penetrate the plasma membrane unless the cells were damaged in some way, although this route may be of relevance to the pinocytic capture of a molecule with a large proportion of hydrophobic groups and thus an affinity for the membrane lipid. The first two points are to some degree investigable by monitoring accumulation of a radiolabelled substrate under conditions that inhibit pinocytosis, such as low temperature or the presence of metabolic inhibitors. Such experiments are described later (Chapter 4) so little will be said of the findings here, only that the tissue association of substrate was not progressive, being completed by the first sample time at 1h, and the quantity of substrate that became tissue associated under these conditions was directly proportional to the extent of binding as had been inferred from the magnitude of the Endocytic Index for the particular substrate. These results indicate strongly that under normal culture conditions indiscriminate extracellular accumulation of radiolabelled substrates does not contribute to the total tissue-associated radioactivity measured. However, there must still be some slight uncertainty, as the modification of conditions could also alter the behaviour of the substrate e.g decreasing the temperature or metabolic energy

supply could affect the binding kinetics themselves or alternatively decrease the surface area of the cell by withdrawal of membrane, causing intracellular spaces to become smaller or larger.

Confirmation of the intracellular location of ^{125}I -PVP and colloidal ^{198}Au following incubation of a yolk sac in medium containing the radiolabelled-substrate has never been achieved. The main difficulty in using a cell fractionation method to investigate the fate of a radiotracer is the small quantities of radioactivity that actually become tissue-associated during incubation. This problem is somewhat reduced for ^{125}I -dBSA as it enters the cell at a much faster rate (by adsorptive pinocytosis) but here there is continual digestion of the substrate and loss of the degradation products, so again the tissue-associated radioactivity is fairly low. It has been shown that ^{125}I -dBSA injected into pregnant rats accumulated in the lysosomal system of the visceral yolk sac (Williams et al., 1971) and a preliminary investigation by Livesey (1979) showed that following incubation of yolk sacs in culture medium containing ^{125}I -dBSA the $[^{125}\text{I}]$ radioactivity distribution in subcellular fractions obtained by differential centrifugation of yolk sac homogenates followed the distribution of acid-proteinase. Goetze et al., (1976) fractionated rat yolk sac at various times after the in vitro uptake of ^{125}I -HSA and then subfractionated the mitochondrial-lysosomal fraction by isopycnic sucrose density gradient centrifugation. They found that it was possible to separate micropinocytic vesicles, primary lysosomes and phagolysosomes according to size and specific weight, and following incubation with radiolabelled substrate the relative distribution of radioactivity had a peak which moved from the light fraction (after 5min) towards

the heavier fraction (20,40min).

The appearance of degradation products after incubation with the protein is indirect evidence that the protein is translocated to the lysosomal system and exposed to lysosomal hydrolases. Of course degradation of the protein at other locations, notably the plasma membrane could be an alternative explanation of these findings, but on examination of the yolk sac plasma membrane no proteases were found (Kenny, unpub.) In addition examination of the culture medium showed that it did not contain fragments of partially degraded ^{125}I -dBSA, only aminoacids and dipeptides (Williams et al., 1975 b), and these observations reinforce the assumption that degradation of ^{125}I -dBSA is not extracellular.

It can be seen that there is much indirect evidence to support the claim that the radiolabelled substrates employed as pinocytic markers for the rat visceral yolk sac do actually perform precisely that function. Ideally a detailed study involving subcellular fractionation of visceral yolk sac after exposure to the three radiotracers should be carried out to confirm unequivocally the intracellular fate of these compounds.

To summarize, it is believed that radiolabelled macromolecules and colloidal ^{198}Au are interiorized by the rat visceral yolk sac epithelium via a pinocytic route. The magnitude of the batch variation in the Endocytic Indices observed for all three probes was sufficiently small to confirm both the suitability of the yolk sac as a pinocytic model and also the reliability of the markers themselves. The stability of the fluid-phase marker ^{125}I -PVP (supplied by the Radiochemical Centre) during both storage and culture was confirmed.

CHAPTER 4.

EFFECT OF TEMPERATURE, METABOLIC AND CYTOSKELETAL
INHIBITORS, CORTICOSTEROIDS, EGTA, THEOPHYLLINE AND
OUABAIN ON PINOCYTOSIS IN THE RAT VISCERAL YOLK SAC.

4.1. INTRODUCTION

The increased oxygen consumption shown by guinea pig leucocytes during phagocytosis of starch granules led to the belief that metabolic energy was essential for endocytosis (Sabarra & Karnovsky, 1959). Later Karnovsky and co-workers showed that particular glycolytic inhibitors such as iodoacetate and sodium fluoride inhibit phagocytosis, whereas inhibitors of oxidative phosphorylation such as cyanide and dinitrophenol do not. They concluded that phagocytosis was an energy consuming process utilizing ATP^{which was not} derived from aerobic oxidation of the tricarboxylic acid cycle intermediates or anaerobic glycolysis. A wide variety of inhibitors of both glycolysis and oxidative phosphorylation have been shown to inhibit pinocytosis. Dinitrophenol and iodoacetate reduce pinocytosis by amoebae (Chapman-Andresen, 1967), inhibit horseradish peroxidase incorporation by kidney tubule cells (Miller et al., 1965) and also limit uptake of several substances by macrophages (Casley-Smith, 1969; Cohn & Parks, 1967; Ehrenreich & Cohn, 1967). Sodium azide, cyanide and fluoride all inhibit horseradish peroxidase uptake by L cells (Steinman et al., 1974).

Although the majority of pinocytic processes would seem to be dependent on metabolism, a few instances have been reported which suggest that pinocytosis cannot be completely blocked by addition of metabolic inhibitors or reduction in temperature. Casely-Smith (1969) showed that carbon, thorium dioxide and ferritin could enter macrophages inside small vesicles even at low temperature or in the presence of large concentrations of inhibitors. To explain such results a category of pinocytosis has been proposed,

called "micropinocytosis" (Allison & Davies, 1974), which is insensitive to cytochalasin B or colchicine and can proceed in the absence of metabolic energy. This mechanism is thought to be an ongoing process involving the capture of small particles in small pinosomes which have a diameter of approximately 70nm. Under this scheme pinocytosis also includes a category "macropinocytosis" which is defined as requiring an energy supply, microfilament/microtubule function and involving vesicles of 0.3-2 μ m in diameter; most pinocytic processes seem of this type (Bowers, 1977; Chapman-Andresen, 1967; Steinman et al., 1974; Miller et al., 1965; Ryser, 1970).

The precise factors that govern the actual rate of formation of pinosomes are still unclear. Certain inorganic salts such as sodium chloride can stimulate pinocytosis in Amoeba proteus (Chapman-Andresen, 1965) but the effect of these ions is certainly not uniform. By analogy to the skeletal muscle and secretory systems, both calcium and cAMP have been suggested as possible regulators of pinocytosis (Stossel, 1977). Recently Hemmaplardh & Morgan (1977) showed that cell membrane calcium is important in regulating the binding of transferrin to reticulocyte receptors, but they found no evidence to suggest that an increase in intracellular calcium affected pinocytic rate. In most cell types a rise in the intracellular cAMP levels causes a decrease in pinocytic rate, although it appears to stimulate pinocytosis in toad bladder cells (see Section 1.1.1.) Another group of substances, the corticosteroids, have been implicated as having a role in the prevention of certain types of pinocytosis during neonatal gut closure in rats, a time

when the uptake of maternal immunoglobulins is thought to stop.

The pinocytic uptake of radiolabelled substrates by the rat yolk sac has been studied extensively (Moore et al., 1974; Williams et al., 1975 a,b; Roberts et al., 1976; Moore et al., 1977; Roberts et al., 1977; Ibbotson, 1978; Livesey, 1979) and it has been found that such substrates enter the tissue either predominantly in the fluid phase, as in the case of ^{125}I -PVP and [^{14}C] sucrose, or alternatively with a large degree of membrane binding as was found for ^{125}I -labelled proteins and colloidal ^{198}Au . Although there is much information regarding the kinetics of uptake of these substrates, little is known of the mechanism of vesicle formation in this tissue.

The rat yolk sac epithelium has a highly microvillous apical border which is active in pinocytosis. It is unlikely that such a membrane could distort sufficiently to capture large particles, so this tissue is not suited to phagocytosis. Vesicles within this tissue show a size range of $0.07\mu\text{m}$ to $2\mu\text{m}$, so the pinocytic uptake mechanism could theoretically be either macropinocytosis or micropinocytosis or a combination of both. In this Chapter the effect of a variety of modifiers on the uptake of ^{125}I -PVP has been examined to try to increase the understanding of the mechanism of pinosome formation in the visceral yolk sac. Some data relating to the uptake of adsorptive substrates ^{125}I -dBSA and colloidal ^{198}Au are included for comparison. All three substrates are potential candidates for micropinocytosis as the maximum particle size of colloidal ^{198}Au is approximately 30nm and the average molecular weights of ^{125}I -dBSA and ^{125}I -PVP are 68000 and 33,000 respectively.

4.2. RESULTS

Quantitation of substrate capture was carried out as described in Chapter 2 and the rate of accumulation when linear with time is expressed as an Endocytic Index. Flasks containing culture medium were placed in water baths pre-set to temperatures in the range 2 - 42°C and the yolk sacs routinely preincubated for 15min. In experiments with chemical modifiers, substrate and modifier were added to the culture simultaneously. Recovery experiments were carried out as described in Section 2.5.

4.2.1. Effect of Temperature and Metabolic Inhibitors

The mean body temperature of ten female Wistar rats was found to be $36.97 \pm 0.18^{\circ}\text{C}$. As the culture temperature deviated from 37°C the Endocytic Index of ^{125}I -PVP fell sharply; below 20°C and above 40°C the progressive accumulation of ^{125}I -PVP ceased. Fig.4.1.shows the relationship between temperature and Endocytic Index and Fig.4.2.shows the decrease in uptake observed as temperature falls. The Q_{10} value between 37° and 30°C was 7.05, whereas the Q_{10} between 37° and 2°C was only 2.5. Fig.4.3.shows the results expressed in the form of an Arrhenius Plot, and the mean activation energy for pinocytosis of ^{125}I -PVP calculated from this graph is 24.1Kcal/mol . Figs. 4.4.and 4.5.show the concentration-dependent effects of 2,4-dinitrophenol and iodoacetate on the uptake of ^{125}I -PVP.

Low temperature, 2,4-dinitrophenol (50µg/ml) and iodoacetate (10/µg/ml), all conditions which abolished the uptake of ^{125}I -PVP, also prevented the pinocytosis of colloidal ^{198}Au (Fig.4.6) and ^{125}I -dBSA(Fig.4.7). Fig.4.7. shows the total pinocytosis

FIG. 4.1. EFFECT OF TEMPERATURE ON THE UPTAKE OF ^{125}I -PVP

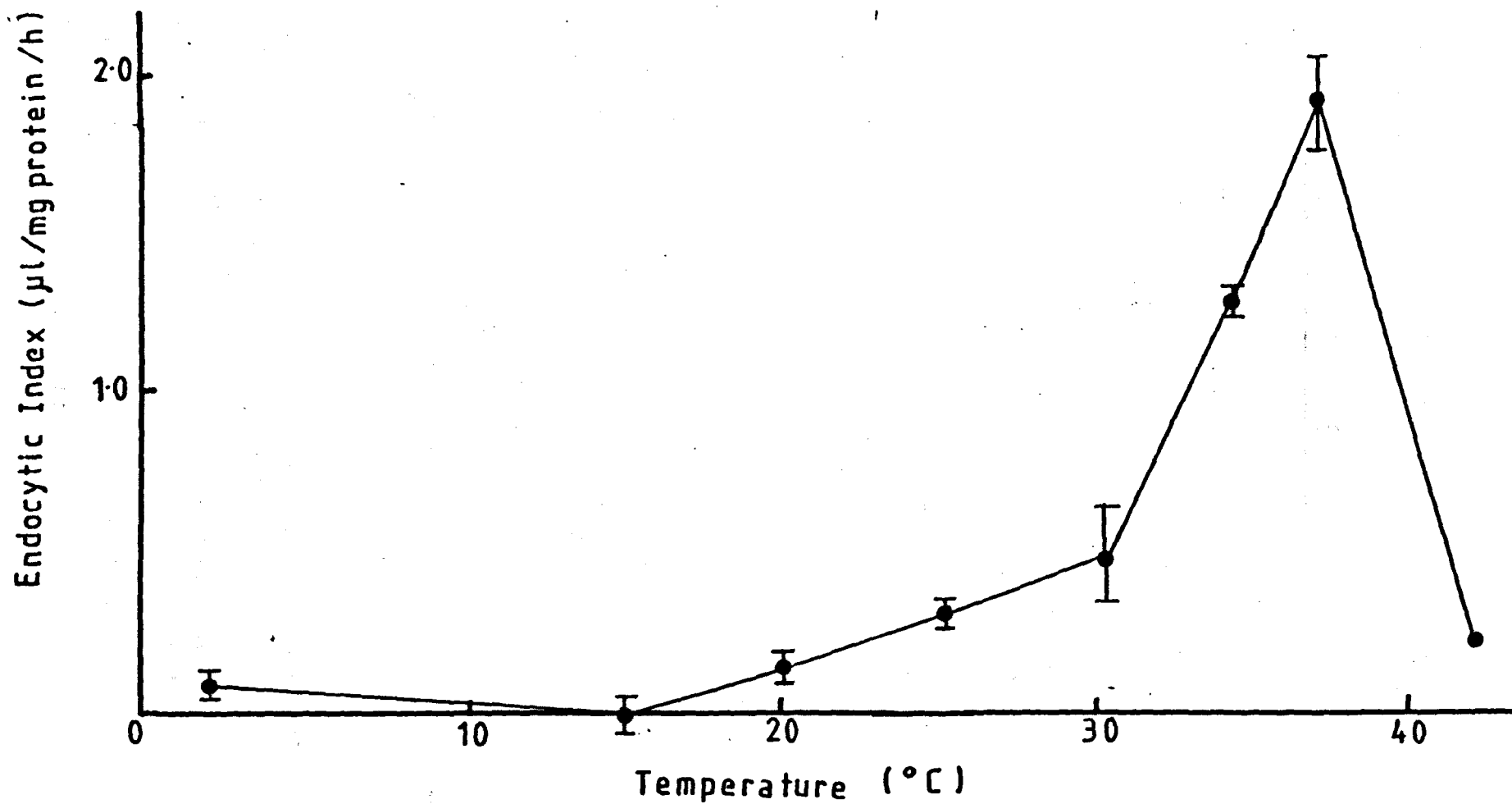


FIG. 4.2. EFFECT OF TEMPERATURE ON THE
UPTAKE OF ^{125}I -PVP

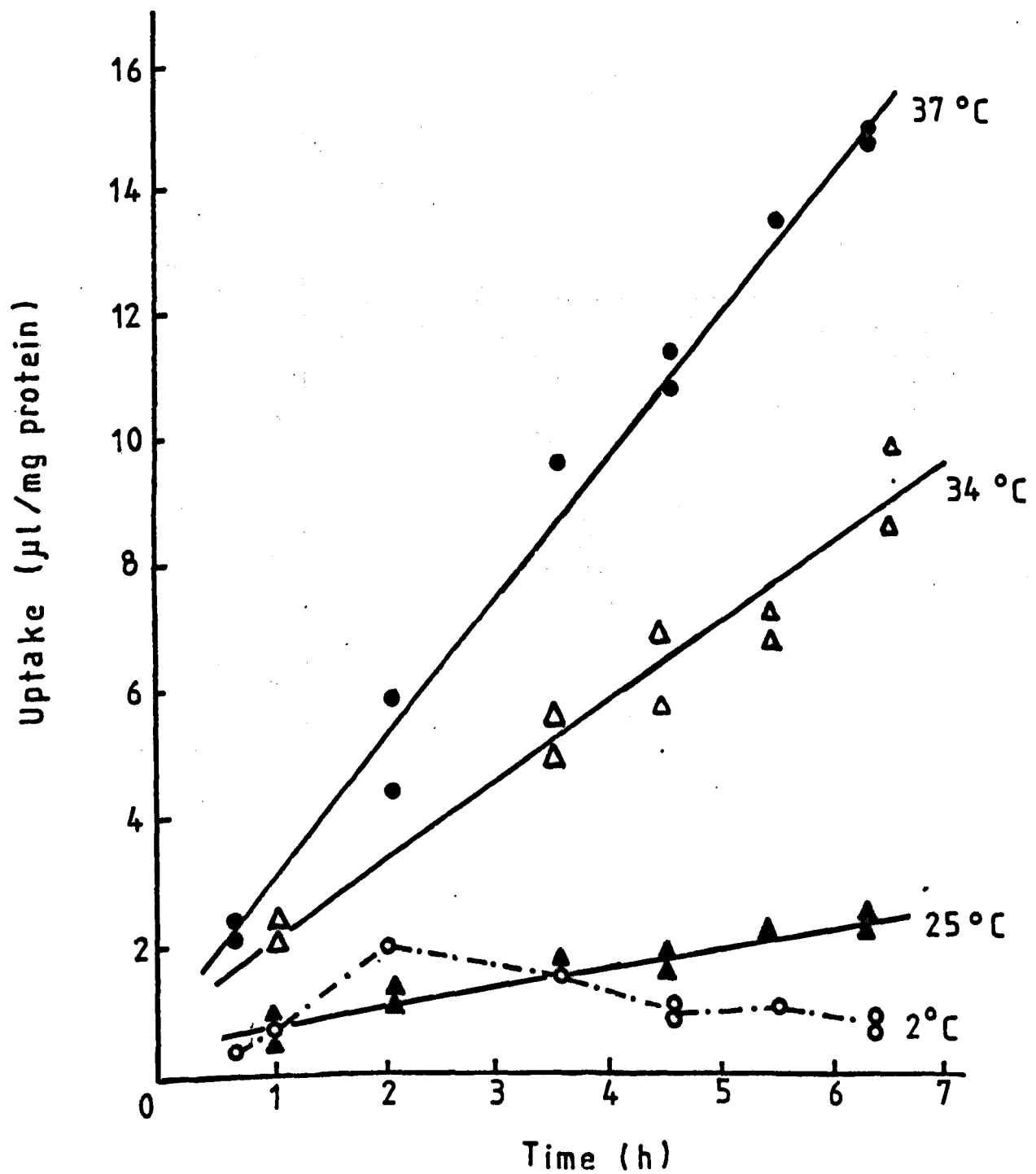


FIG. 4.3. ARRHENIUS RELATIONSHIP BETWEEN
TEMPERATURE AND THE ENDOCYTIC INDEX
OF ^{125}I -PVP

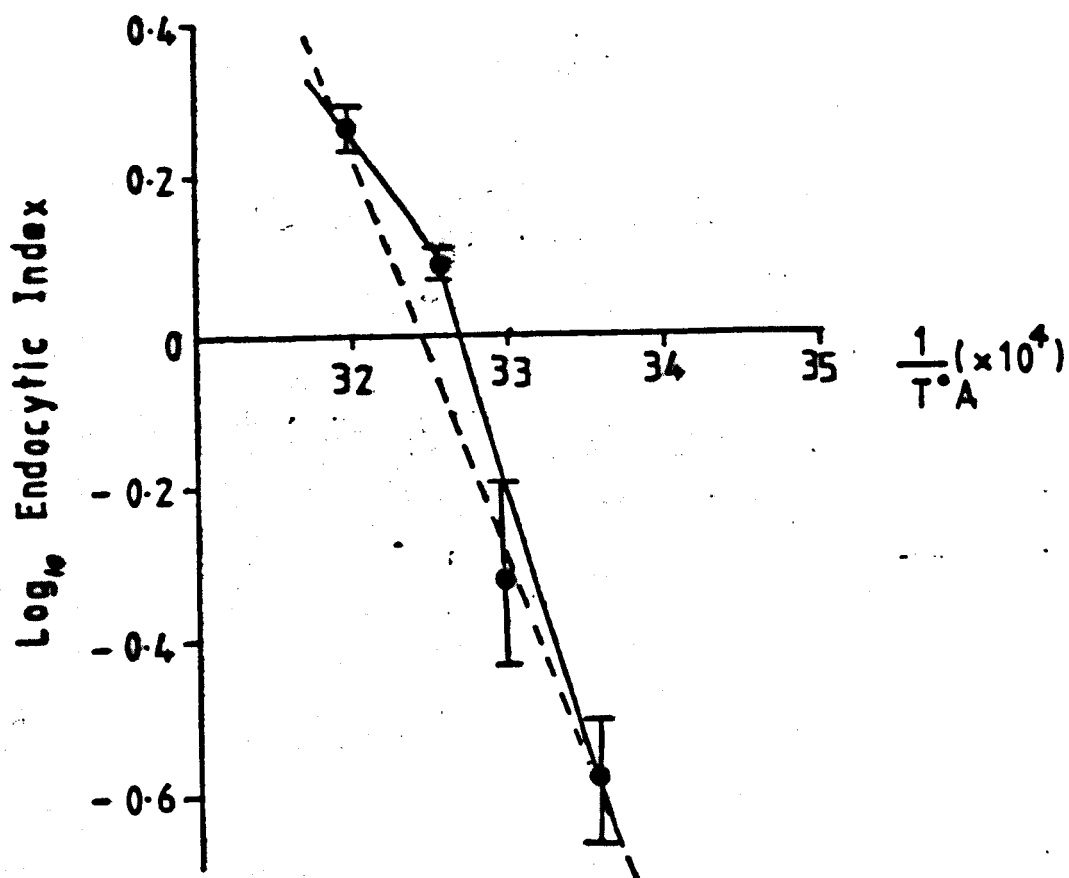


FIG.4.4. EFFECT OF 2,4 DINITROPHENOL ON THE UPTAKE

OF ^{125}I -PVP

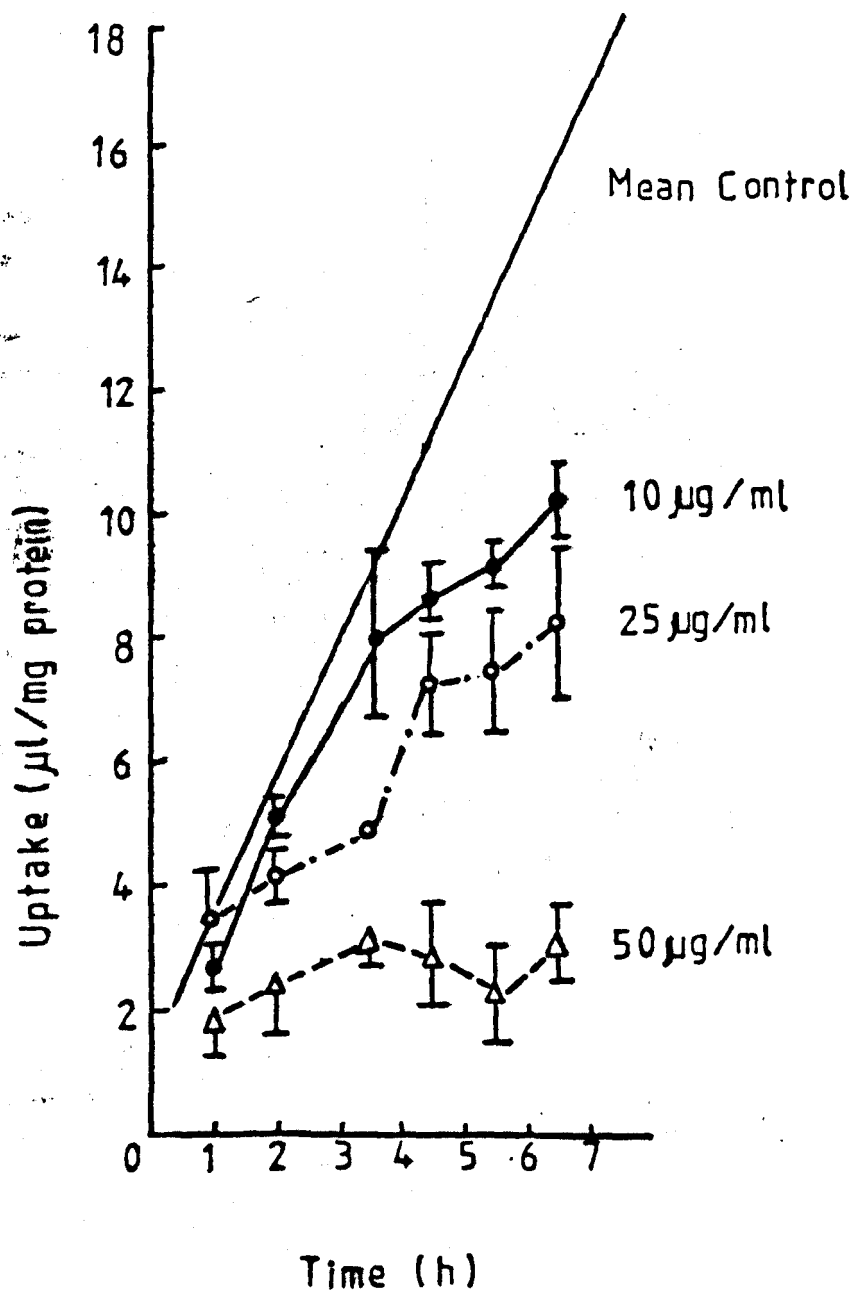


FIG. 4.5. EFFECT OF MONOiodoacetate ON THE

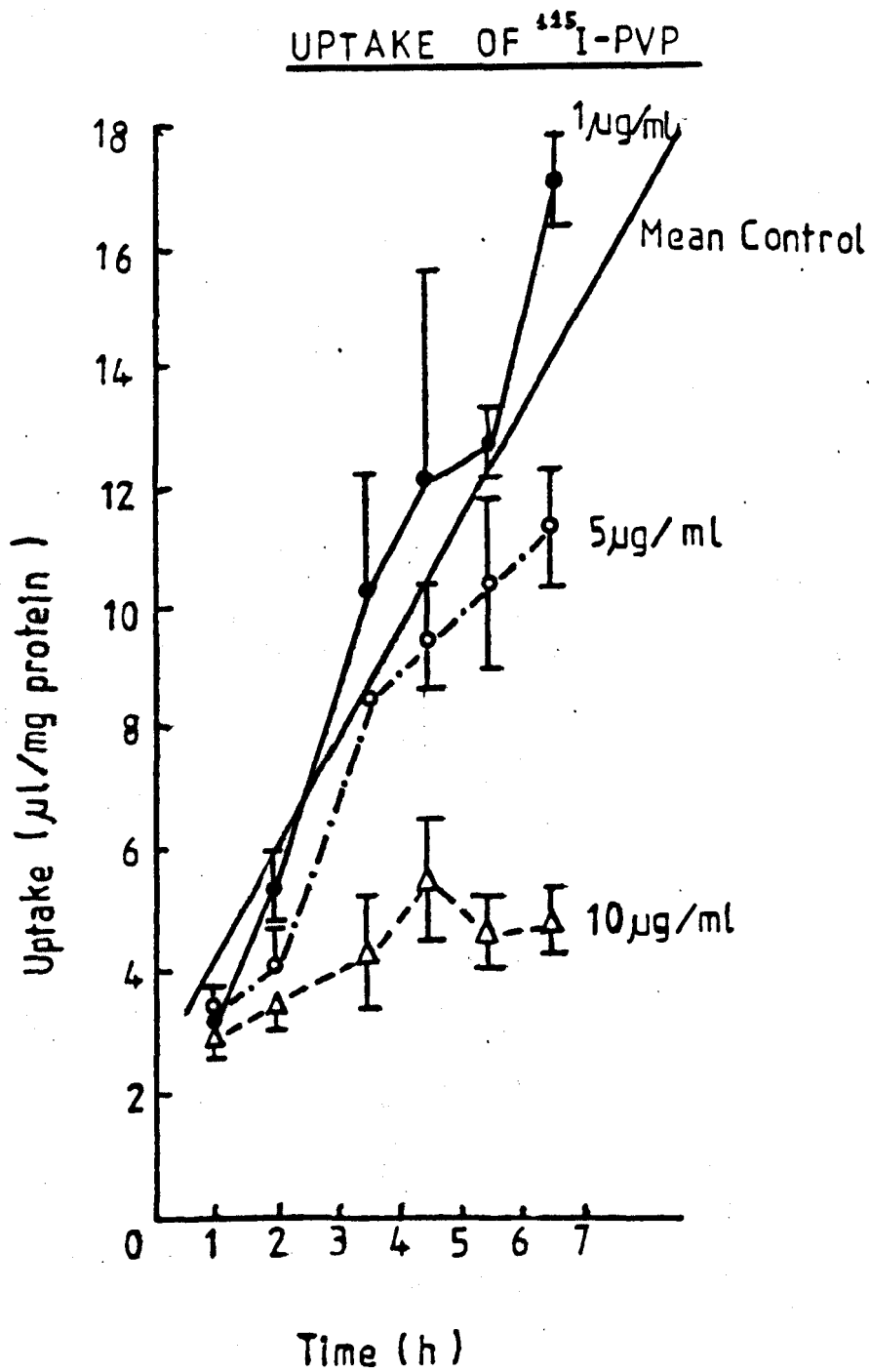
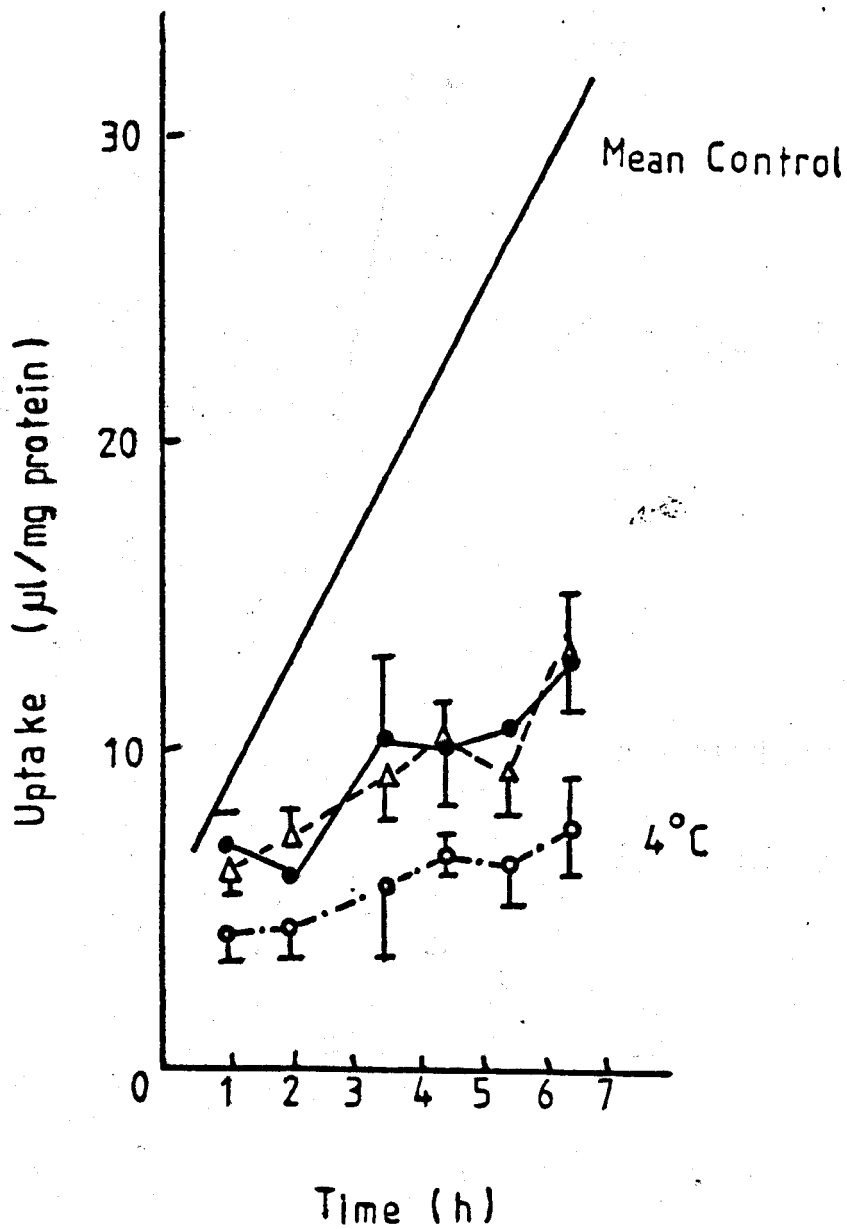
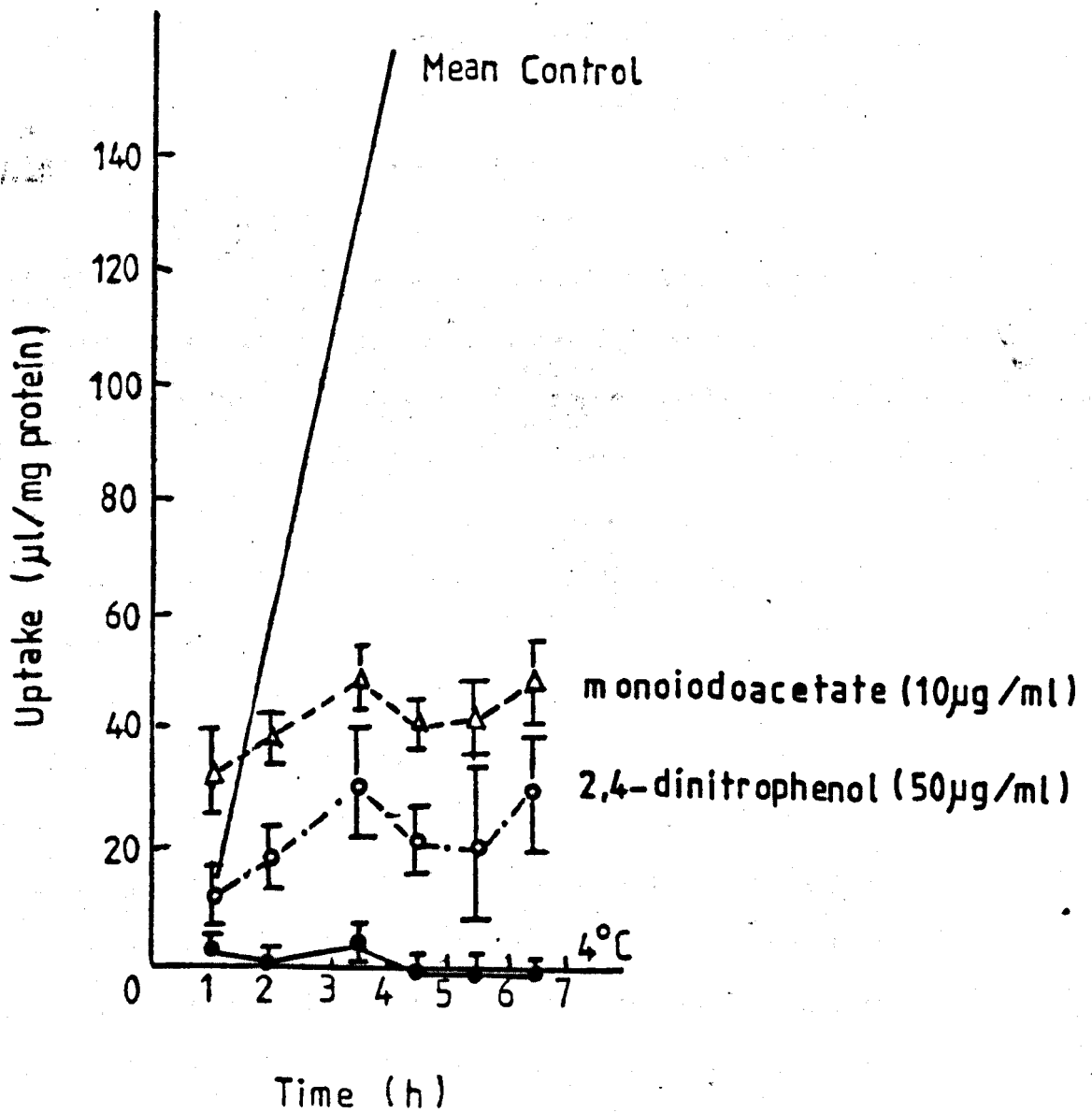


FIG. 4.6. EFFECT OF METABOLIC INHIBITORS AND LOW
TEMPERATURE ON THE UPTAKE OF COLLOIDAL ^{199}Au



●—● monolodoacetate (10 $\mu\text{g/ml}$)
Δ---Δ 2,4-dinitrophenol (50 $\mu\text{g/ml}$)

FIG. 4.7. EFFECT OF METABOLIC INHIBITORS AND LOW
TEMPERATURE ON THE UPTAKE OF 125 I-dBSA



of ^{125}I -dBSA, the tissue level and TCA-soluble radioactivity being summed (see Section 2.3); here the inhibitors effectively prevented uptake and, as expected, there was no progressive release of acid-soluble radioactivity. Consequently the uptake values given here represent almost entirely tissue-associated radioactivity and are therefore directly comparable with the data on the non-digestible substrates.

To ensure that the conditions employed had no effect on the TCA-soluble level within the ^{125}I -dBSA preparation and hence bias the uptake values calculated, flasks were set up containing 2,4-dinitrophenol (50 $\mu\text{g}/\text{ml}$), moniodoacetate (10 $\mu\text{g}/\text{ml}$) at 3°C or 37°C and the percentage solubles estimated after 6h incubation. The results are shown in Table 4.1 and it can be seen that the values obtained in the presence of 2,4-dinitrophenol and at 3°C (0.55% \pm 0.08 and 0.48% \pm 0.05) respectively were not significantly different for the value obtained at 37°C (0.57% \pm 0.07). In the presence of moniodoacetate the percentage solubles was approximately double at 1.01 \pm 0.08%, but this value was still acceptably low.

It can be seen from Fig.4.4. to 4.7. that some radioactivity was always found in association with the tissue, even under conditions where progressive uptake has ceased. This presumably represented occluded material not removed by the washing procedure and also substrate bound firmly to, but not internalized by the yolk sac. Some contribution from residual pinocytic activity in the first hour of incubation was possible in the case of chemical inhibitors, but unlikely in the 3°C incubations, where

TABLE 4.1.

THE EFFECT OF VARIOUS CULTURE CONDITIONS ON THE PERCENTAGE TCA SOLUBLERADIOACTIVITY IN A PREPARATION OF ^{125}I -dBSA.

| <u>Experiment</u> | <u>Culture Conditions</u> | | | |
|-------------------|---------------------------|---------------------------------------|------------------------------------|-----------------|
| | 37° Control | 37° + 2,4-dinitrophenol (50 µg/ml) | 37° + Moniodoacetate (10 µg/ml) | 3°C |
| 1 | 0.74 | 0.44 | 1.06 | 0.44 |
| 2 | 0.55 | 0.44 | 0.98 | 0.52 |
| 3 | 0.52 | 0.65 | 0.91 | 0.43 |
| 4 | 0.52 | 0.53 | 1.14 | 0.48 |
| 5 | 0.53 | 0.52 | 0.97 | 0.49 |
| 6 | 0.53 | 0.52 | 0.98 | 0.50 |
| 7 | 0.57 | 0.52 | 1.04 | 0.54 |
| 8 | 0.57 | 0.65 | - | 0.41 |
| Mean \pm S.D. | 0.57 \pm 0.07 | 0.55 \pm 0.08 | 1.01 \pm 0.08 | 0.48 \pm 0.05 |

Figures represent the percentage TCA soluble activity measured in medium 199 containing 10% calf serum after culture for 6h.

the preincubation period was long enough to bring the tissue to equilibrium.

4.2.2. Effect of Cytoskeletal Inhibitors

Colchicine at 1,5, or 10 μ g/ml decreased the rate of uptake of 125 I-PVP (Fig.4.8). All three concentrations affected uptake to a similar extent, the mean Endocytic Indices being 0.57, 0.35 and 0.18 μ l/mg protein/h respectively. The mean control Endocytic Index for this set of data was 2.08 μ l/mg protein/h. Cytochalasin B at 1 μ g/ml had no effect on the uptake of 125 I-PVP but 5 μ g/ml, and 10 μ g/ml uptake seems to cease after 3.5h incubation, whereas the higher concentration of 10 μ g/ml shows a slower but more or less progressive accumulation over the 6.5h culture period, (Fig.4.9).

4.2.3 Recovery Pinocytosis by Yolk Sacs after Incubation with 2,4-Dinitrophenol, Moniodoacetate and Colchicine

It can be seen from Fig. 4.10. that washing the yolk sacs in warm medium (37°C) and 10% calf serum caused no change in the rate of uptake of 125 I-PVP. The experiment in which the yolk sacs were washed had a lower rate of uptake throughout than that in which the yolk sacs were untreated. All three modifiers, 2,4-dinitrophenol (50 μ g/ml), moniodoacetate (10 μ g/ml) and colchicine (10 μ g/ml) caused inhibition of 125 I-PVP uptake consistent with that reported earlier, except in the case of moniodoacetate which showed a small but apparently linear accumulation during the first 3.5h; however, there were only four samples taken during this time. Following washing and removal of modifier there was no apparent return to a normal rate of uptake (Fig.4.11).

FIG.4.8. EFFECT OF COLCHICINE ON THE UPTAKE
OF 125 I-PVP

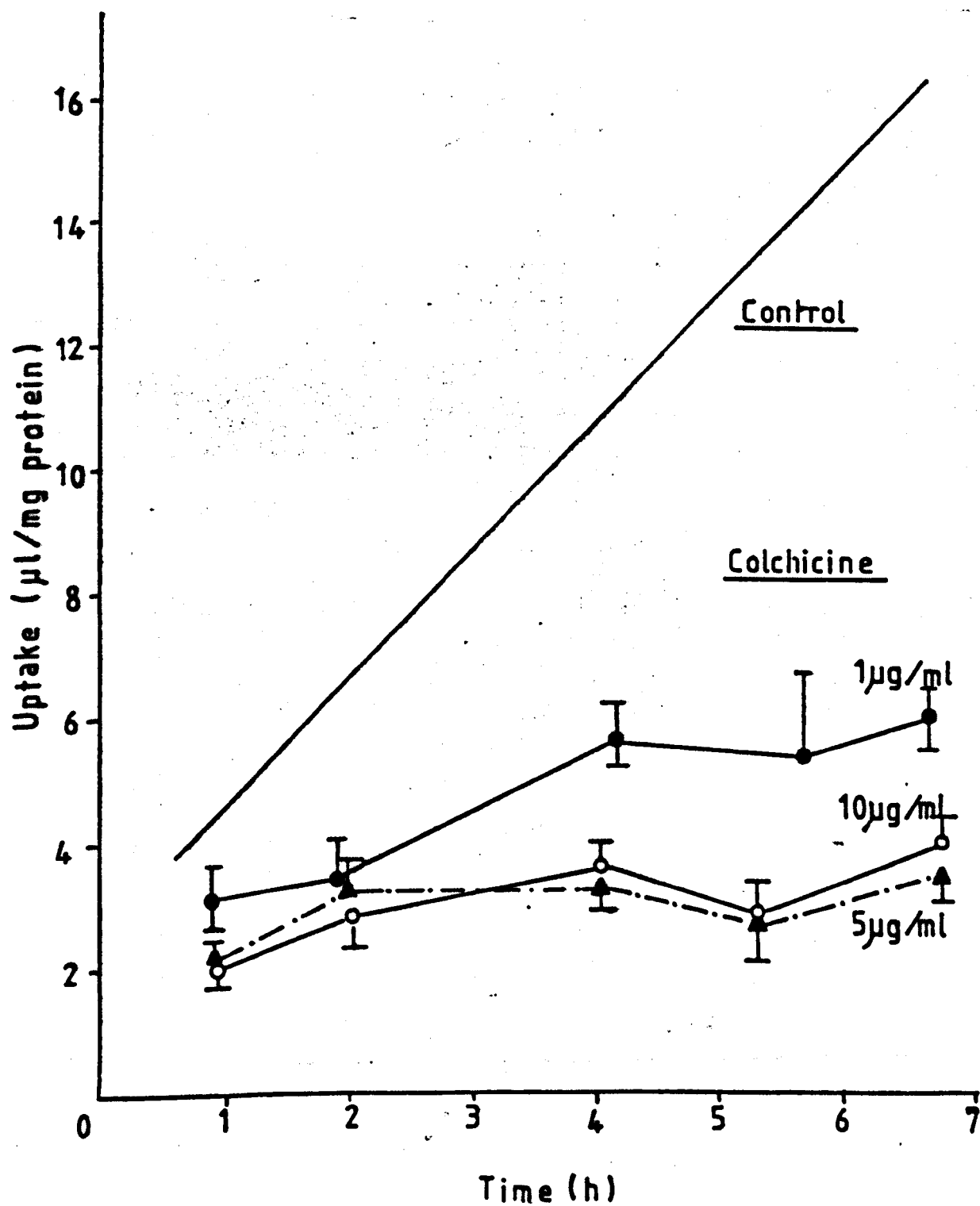


FIG.4.9. EFFECT OF CYTOCHALASIN B ON THE UPTAKE
OF ^{125}I -PVP

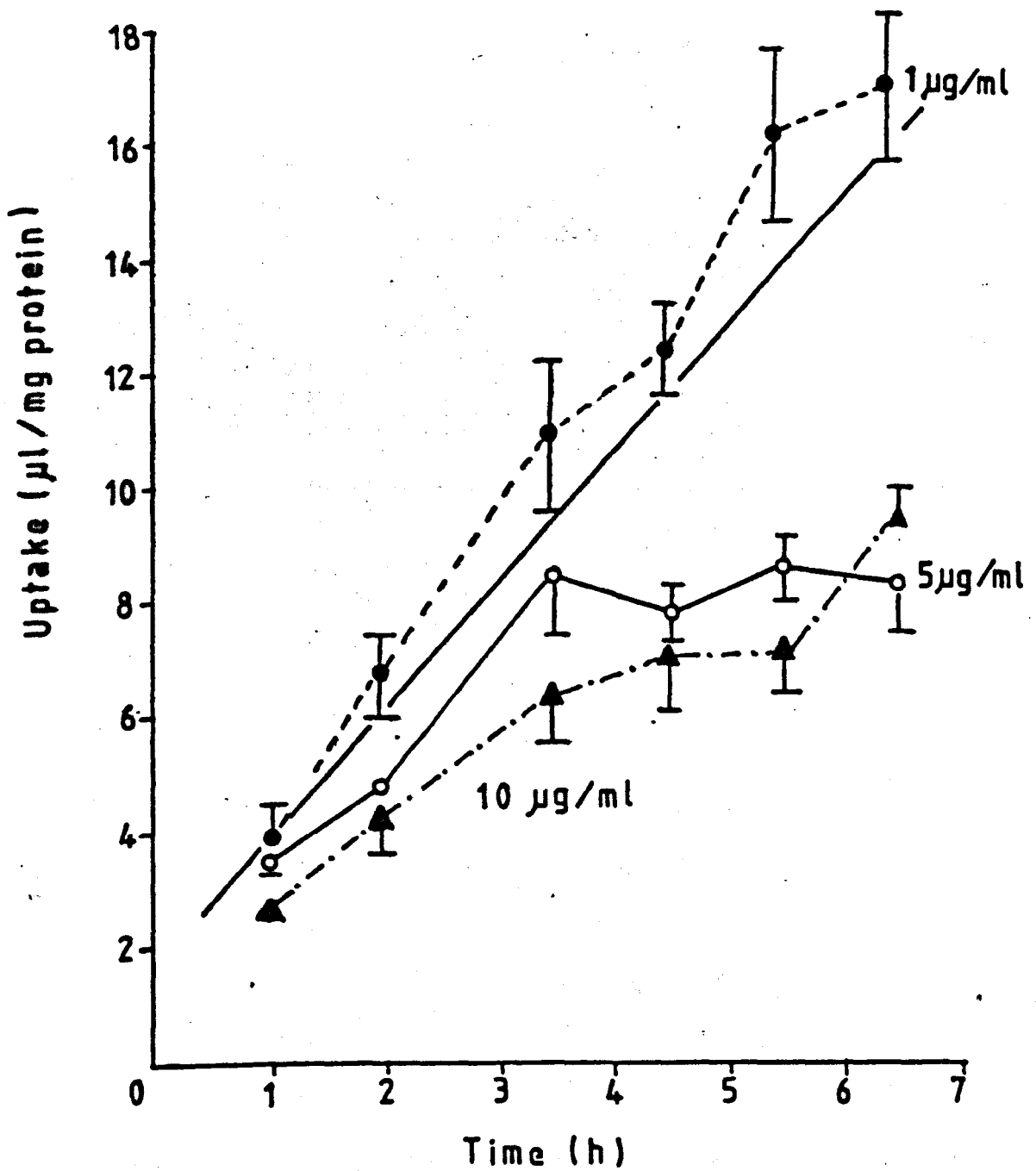
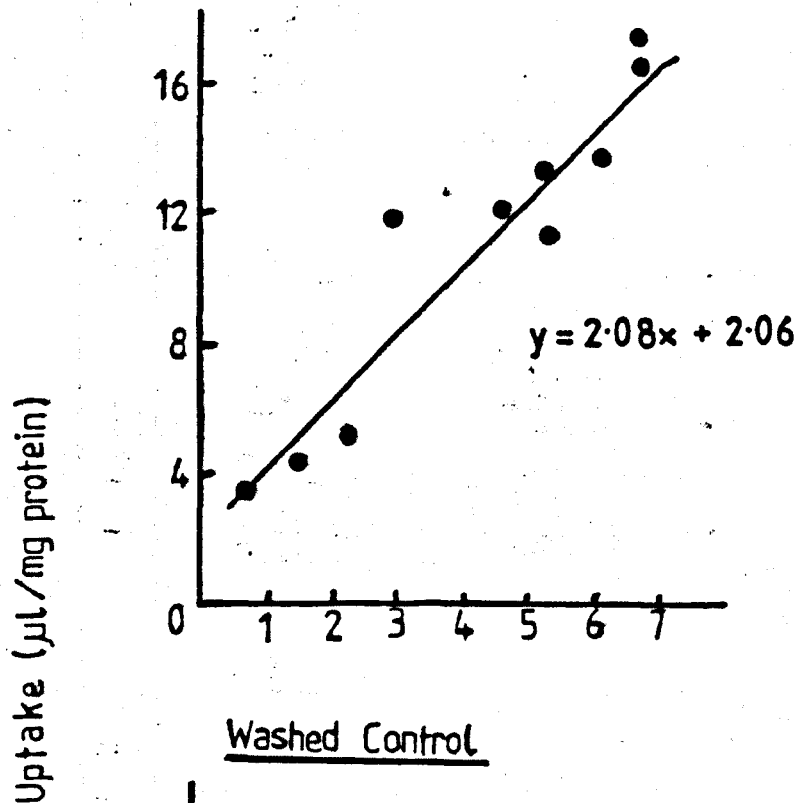


FIG.4.10. EFFECT OF WASHING (3x2min) ON PINOCYTOSIS

OF ^{125}I -PVP [These experiments are controls for
those shown in Fig.4.11.]
Untreated Control



Washed Control

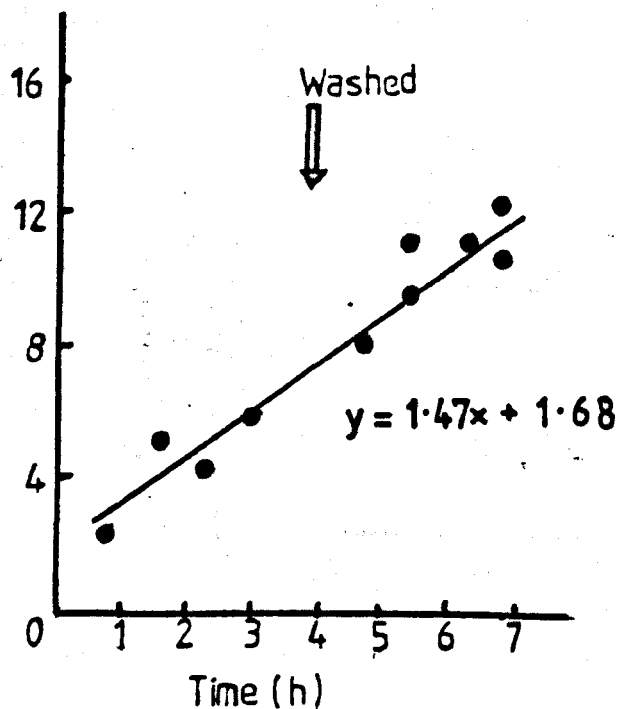
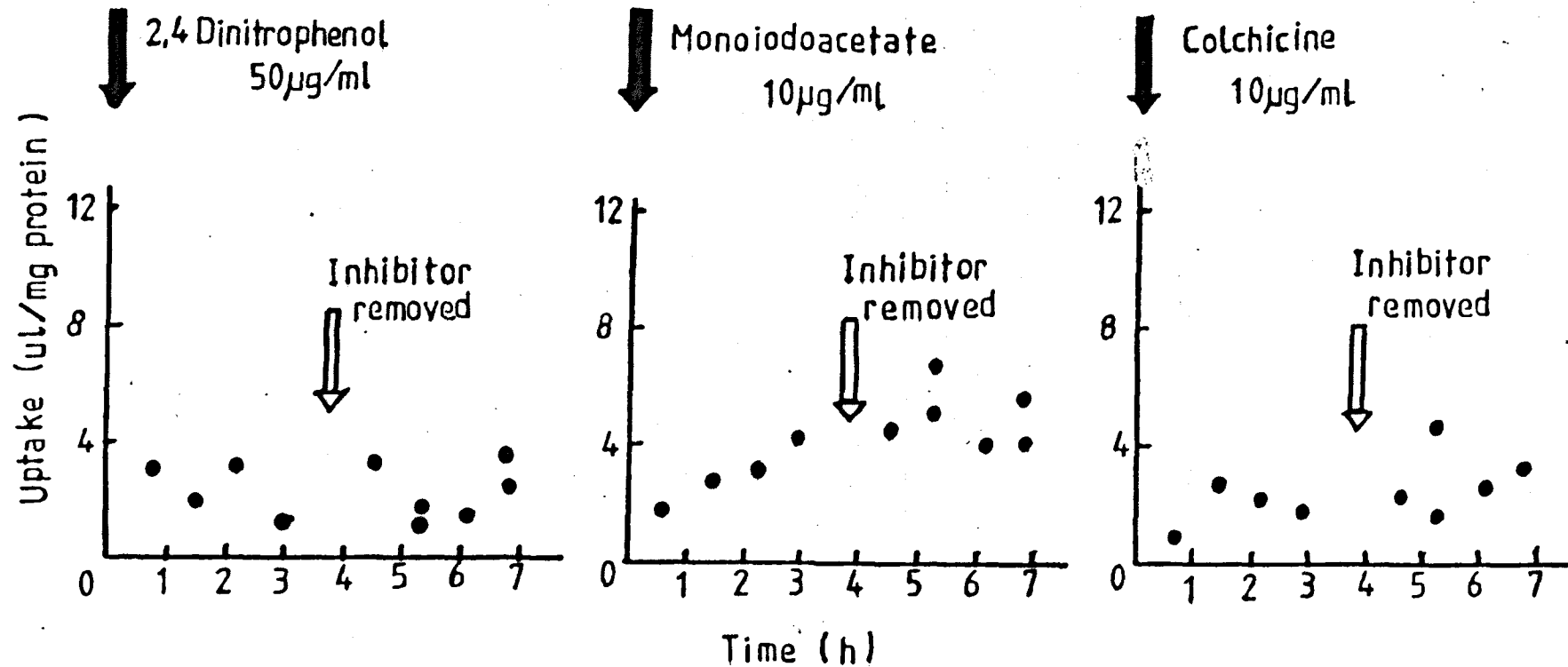


FIG. 4.11. THE INABILITY OF THE YOLK SAC TO RESUME PINOCTOSIS
AFTER EXPOSURE TO A VARIETY OF INHIBITORS



4.2.4. Effect of EGTA, Theophylline and Ouabain

When 5mM EGTA was added to the culture medium uptake of ^{125}I -PVP ceased (Fig. 4.12). This inhibitory effect was abolished if 5mM CaCl_2 and 5mM EGTA were both present. The addition of 5mM EGTA and 5mM MgCl_2 caused total inhibition of ^{125}I -PVP uptake.

Theophylline inhibited pinocytosis in a concentration-dependent manner (Fig. 4.13). Ouabain at 10 $\mu\text{g/ml}$ slightly stimulated uptake of ^{125}I -PVP whereas at 100 $\mu\text{g/ml}$ it was markedly inhibitory. All the effects produced linear changes in the accumulation of ^{125}I -PVP and the results are shown in Fig. 4.14.

4.2.5. Effect of Corticosteroids

Preliminary experiments were carried out to investigate the effect of corticosteroids on the uptake of ^{125}I -PVP. Fig. 4.15. shows that corticosterone (10 $\mu\text{g/ml}$) prevented the uptake of ^{125}I -PVP after 4.5h, having no effect during the initial stages of incubation. Fig. 4.16 shows that cortisone acetate (10 $\mu\text{g/ml}$) had no effect on the rate of ^{125}I -PVP accumulation over 6.5h.

FIG. 4.12. EFFECT OF EGTA ON THE UPTAKE OF ^{125}I -PVP

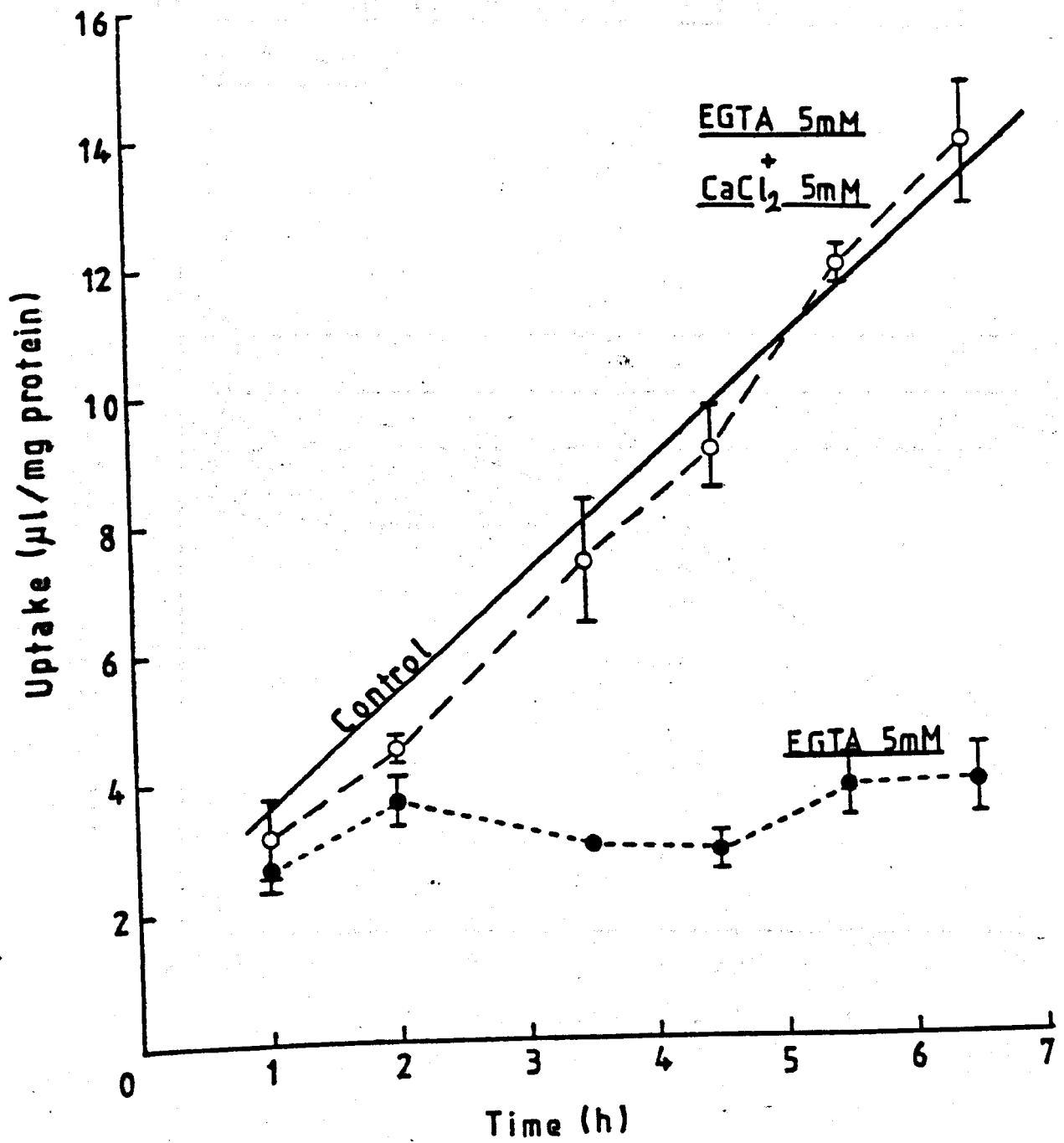


FIG.4.13.EFFECT OF THEOPHYLLINE ON THE UPTAKE
OF ^{125}I -PVP

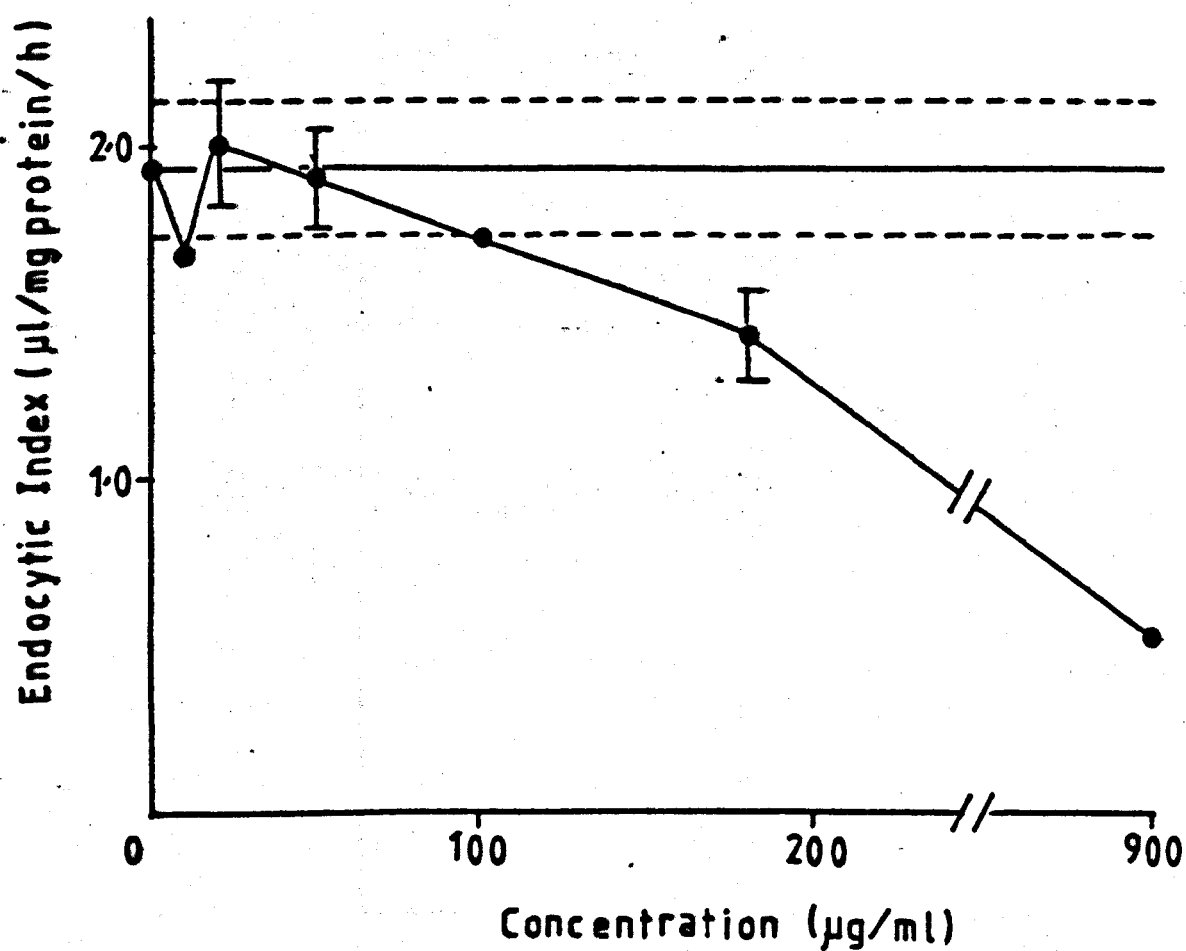


FIG. 4.14. EFFECT OF OUABAIN ON THE UPTAKE
OF ^{125}I -PVP

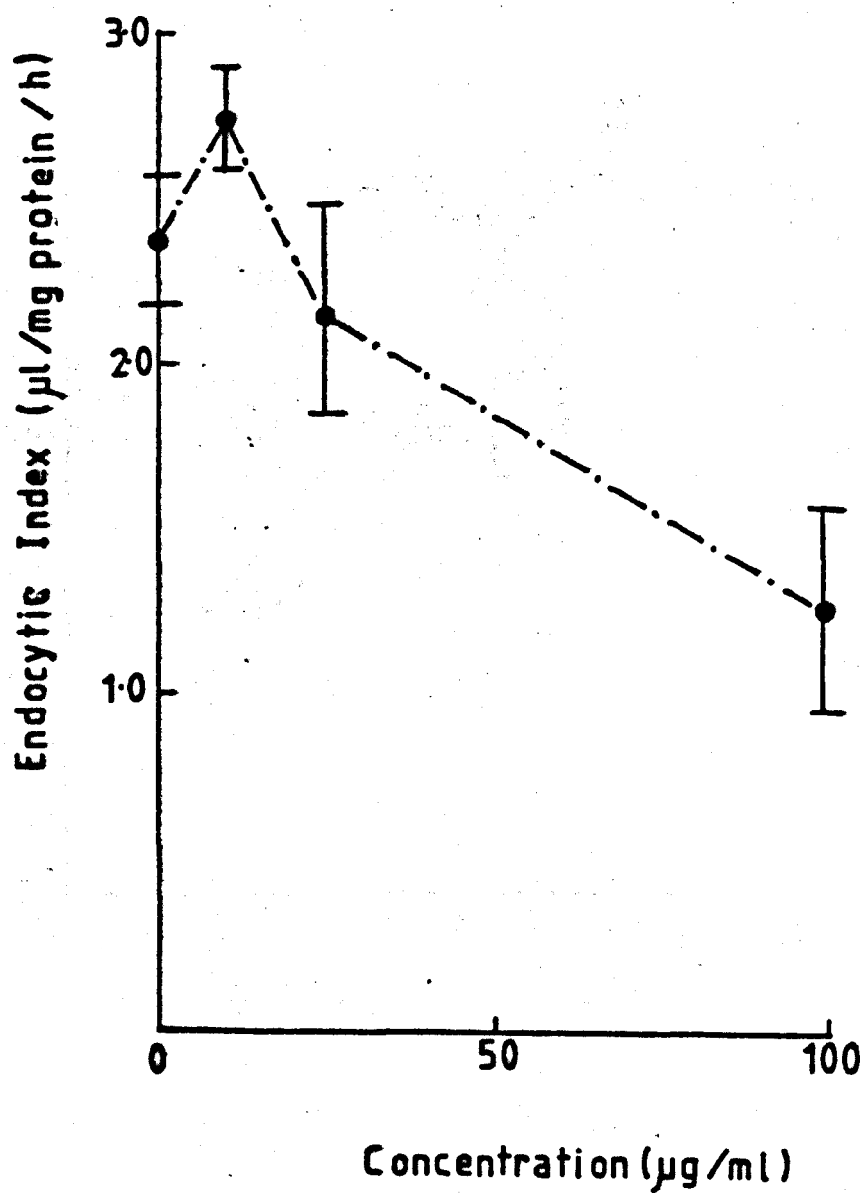


FIG. 4.15. EFFECT OF CORTICOSTERONE (12.5 $\mu\text{g}/\text{mL}$) ON

THE UPTAKE OF ^{125}I -PVP

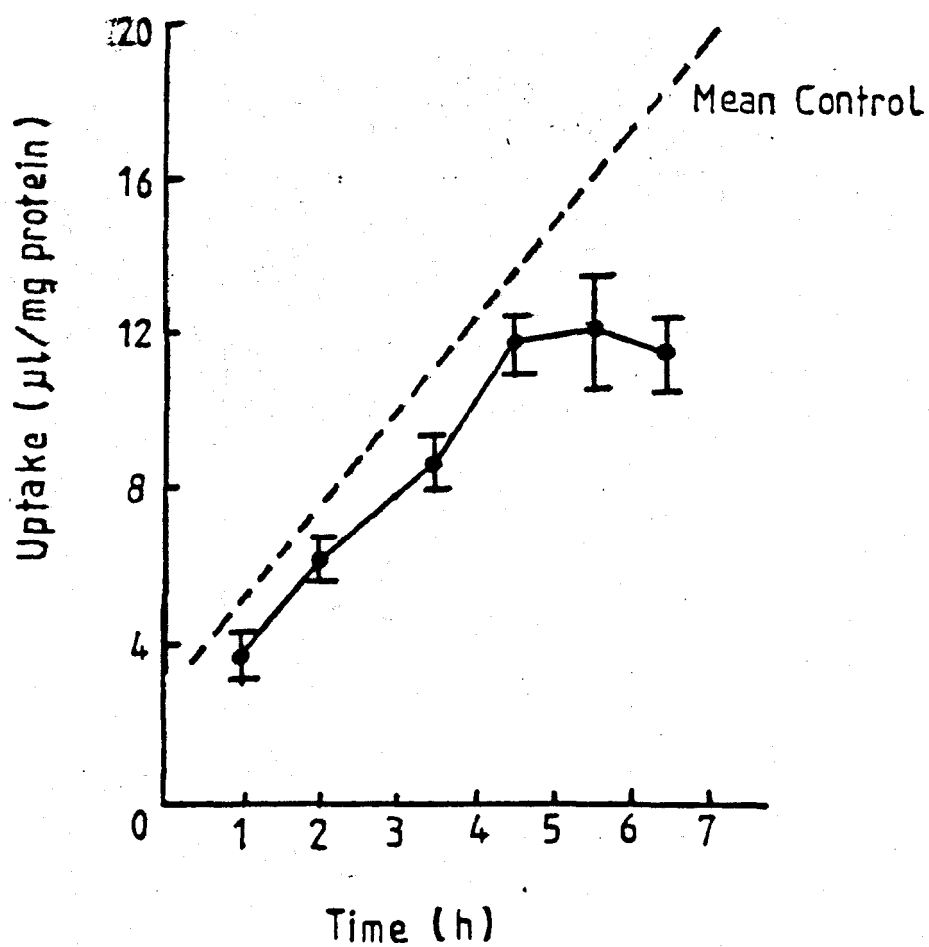
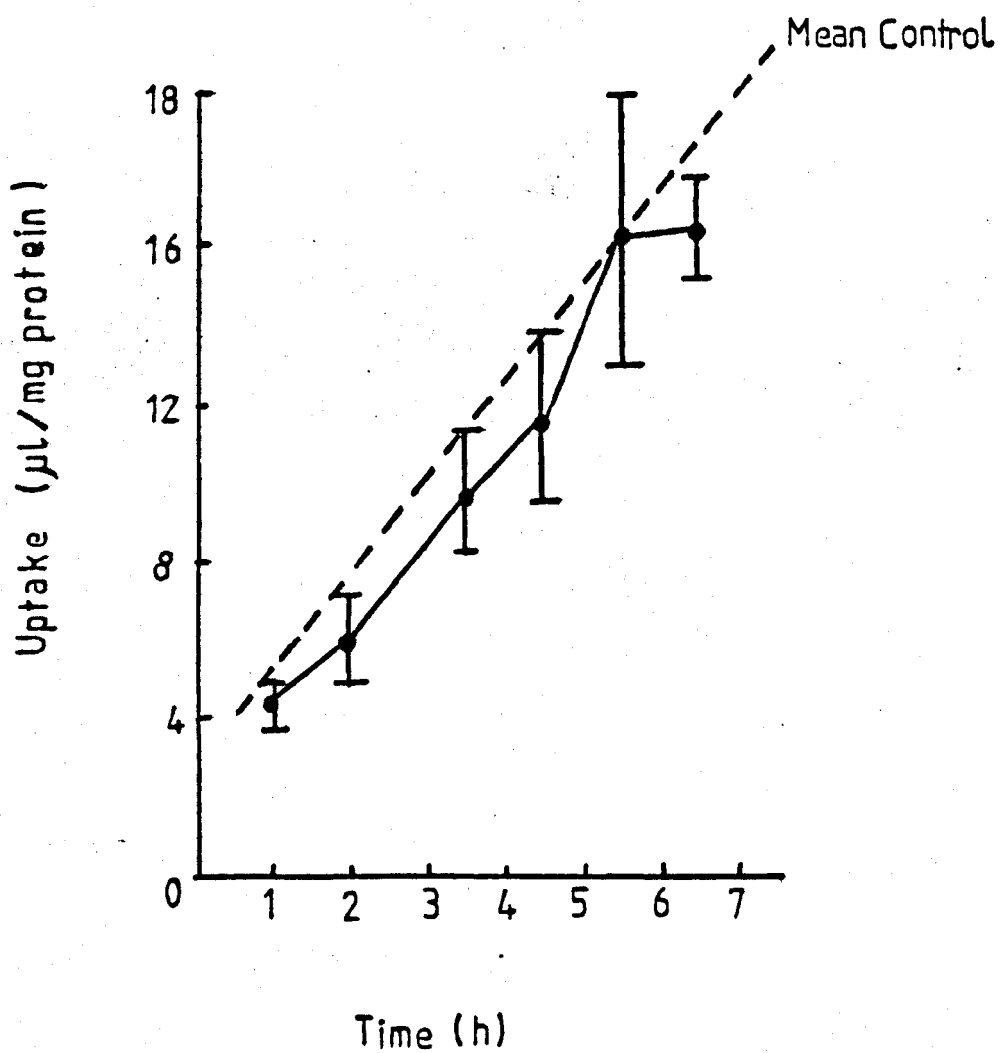


FIG.4.16. EFFECT OF CORTISONE ACETATE (35 μ g/ml)
ON THE UPTAKE OF 125 I-PVP



4.3. DISCUSSION

Examination of the uptake of ^{125}I -PVP over a wide range of temperatures showed that even small deviations from the physiological norm led to a drastic reduction in the rate of capture. A decrease in temperature from 37° to 30°C decreased the Endocytic Index by approximately 75% and below 20°C progressive accumulation ceased. It is interesting to note that at very low temperature (3°C) the uptake graph displayed a characteristic shape (Fig.4.2) with slightly higher tissue-associated activity during the first two hours than during the rest of the incubation. If these uptake values do represent bound radioactivity this phenomenon would be explicable if the yolk sac surface area gradually reduced during the initial stages of an incubation; such shrinkage might be expected at low temperature.

Lowering of temperature exerts a twofold effect. Firstly the supply of metabolic energy will be reduced and secondly the mobility of the membrane components will be altered. Any alteration in membrane fluidity could certainly influence membrane invagination during pinosome formation, so it is difficult to assess which of these two effects is of greater importance in determining the effects of low temperature. Between 37° and 30°C the reduction in uptake of ^{125}I -PVP has a Q_{10} value of 7.05, which is considerably higher than the Q_{10} value of 2 normally associated with metabolic energy dependence. This value is also markedly higher than the value of 3.3 found in studies on pinocytosis in mouse peritoneal macrophages (Cohn, 1966) and that 2.7 reported for pinocytosis in L cells (Steinman et al., 1974). Those measurements were,

however, made over larger temperature ranges; the Q_{10} between 2° and 37°C in these experiments is only 2.5, but this value does not adequately describe the temperature dependence actually observed.

An Arrhenius plot was used as an alternative method of describing the relationship between temperature and rate of pinocytosis (Fig.4.3). Q_{10} values are more correctly applied to single enzymatic reactions, whereas the examination of the response of a total physiological process to temperature change obviously involves the interaction of many different reactions. In the past the Arrhenius equation has been used to describe many complex physiological processes such as heart rate and respiratory movements, so it was considered suitable for application to the process under investigation here. Hoar (1973) discusses the Arrhenius plot in some detail. It can be seen from Fig. 4.3. that the data can be interpreted either as a single line displaying scatter or as a pair of lines of different gradient which intersect at some critical temperature. As this plot consists of only four points, it is difficult to make meaningful deductions, although it has been suggested that sharp changes in an Arrhenius plot can represent a change from one master reaction to another. The activation energies calculated from the gradients displayed on the graph are as follows:-

$$\text{Activation Energy } (37 - 34^{\circ}\text{C}) = 12.9 \text{ Kcal/mol}$$

$$\text{Activation Energy } (34 - 25^{\circ}\text{C}) = 31.1 \text{ Kcal/mol}$$

$$\text{Activation Energy } (37 - 25^{\circ}\text{C}) = 24.1 \text{ Kcal/mol}$$

The last two values estimated here are relatively high as respiratory and cardiac rhythms have an activation energy only in the region

16 - 17 Kcal/mol. Steinman et al., (1974) calculated an activation energy of 17.6 Kcal/mol for the uptake of horseradish peroxidase by fibroblasts and this was derived from a linear relationship between Log rate of uptake and the reciprocal of temperature in $^{\circ}\text{A}$ between 10°C and 30°C ; again the estimated values for yolk sac seem to be somewhat higher. Recently activation energies have been estimated for the pinocytosis of horseradish peroxidase and phagocytosis of antibody-coated erythrocytes by mouse peritoneal macrophages; these values were 17 - 25 Kcal/mol, and 54 - 90 Kcal/mol respectively (Mahoney et al., 1977).

The introduction of an inhibitor of glycolysis or an uncoupler of oxidative phosphorylation into the culture medium caused considerable inhibition of uptake of all three substrates. Moniodoacetate at $10\mu\text{g/ml}$ and 2,4-dinitrophenol at $50\mu\text{g/ml}$ completely abolished pinocytic uptake and, since uptake of all three substrates was inhibited to a similar extent, there was no evidence for different substrate-specific capture mechanisms, the results being compatible with the postulate that these substrates are internalized in a single vesicle type with differing degrees of membrane adsorption. Inhibition of pinocytosis by moniodoacetate and 2,4-dinitrophenol has been described in several other experimental systems (Bowers, 1977; Cohn, 1966; and Munthe-Kaas, 1977) and the concentrations found effective here are in general agreement with those reported as inhibitory and non-toxic to cultured cells. The inhibition caused by these substances was not found to be reversible, when the yolk sacs were washed and returned to medium 199 containing only 10% calf and radiolabelled substrate (Fig.4.11).

Under extreme conditions where progressive pinocytic uptake was

prevented, some radioactivity was always found associated with the tissue. The levels of residual activity are 2-4 $\mu\text{l}/\text{mg}$ protein for ^{125}I -PVP, 20-40 $\mu\text{l}/\text{mg}$ protein for ^{125}I -BSA and 5-8 $\mu\text{l}/\text{mg}/\text{protein}$ for colloidal ^{198}Au , values that correlate with the extent of binding to the tissue as revealed by their Endocytic Indices (see Chapter 3).

Low concentrations (less than 10 $\mu\text{g}/\text{ml}$) of cytochalasin B are consistently found to inhibit phagocytosis (Munthe-Kaas, 1976; Klaus, 1973), but the reported effects on pinocytosis are contradictory. Wills et al., (1972) reported that cytochalasin B (10 $\mu\text{g}/\text{ml}$) did not inhibit pinocytosis by macrophages, whereas Figura & Kresse (1974) demonstrated inhibition of the uptake of lysosomal enzymes by cultivated skin fibroblasts at a similar concentration. The finding that cytochalasin B (5 or 10 $\mu\text{g}/\text{ml}$) had little effect on the pinocytosis of ^{125}I -PVP in the initial phase of a 6h culture period, but almost totally suppressed uptake after 3.5h may contain the key to resolving the apparent contradictions. Colchicine, which is known to impair microtubular function, also inhibited the uptake of ^{125}I -PVP non-reversibly (Fig.4.11.) but in this case uptake was linear with time. All concentrations of colchicine investigated reduced uptake to approximately 20% of the control value, suggesting that this proportion of the pinocytic uptake by the yolk sac is not dependent on the microtubular system. It is not surprising that cytoskeletal inhibitors affect pinocytosis in this way. Goldman and Follett (1969) suggested that the bundles of microfilaments seen beneath the plasma membrane are the basis of a contractile system that facilitates membrane movement, such as is seen during vesicle formation and that microtubules form the cytoskeletal support system, to which the

filaments attach and against which they contract. Reaven & Axline (1972) reached similar conclusions after examining resting and phagocytosing macrophages cultivated on glass.

As the uptake of all three substrates showed a marked sensitivity to metabolic inhibition and are partially inhibited by colchicine and inhibited by cytochalasin B at 5 $\mu\text{g/ml}$, it would appear that pinocytosis in the yolk sac is a mechanism other than micropinocytosis as defined by Allison & Davies (1974). (Incidentally, this contrasts with evidence that in some other tissues (Munthe - Kaas, 1977; Davies et al., 1973) where it is deduced that colloidal ^{198}Au is taken up at least in part by micropinocytosis). However, it should be noted that small, 70-100nm diameter vesicles are frequently visible in electron micrographs of yolk sac (see Fig. 1.4.).

Classification of the mechanism of substrate capture by the rat yolk sac is thus extremely difficult if vesicle size, substrate size, and metabolic/cytoskeletal requirements are used as criteria. These results and a consideration of other published work lead to the conclusion that, while discrete categories of pinocytosis may exist, insufficient data are at present available to define the precise criteria necessary for a classification scheme. It is particularly difficult to envisage any mode of pinocytic uptake that could be completely self-powered, no matter how small the pinosome employed for transport. Casley-Smith & Chin (1971) suggested that small vesicles could acquire sufficient energy from their Brownian motion to traverse cells and fuse with the plasma membrane, but this does not explain how the plasma membrane obtains sufficient energy to invaginate during the first stages of pinosome formation. Nevertheless, vesicles containing substrates have been observed in

rabbit peritoneal macrophages maintained at low temperatures (Casley-Smith, 1969) and radiotracers have shown a small but progressive accumulation in trophoblast cells (Contractor & Kraukauer, 1976), also at low temperature. Are such observations truly pinocytosis? Substrate-containing vesicles seen after exposure to the substrate at low temperatures might simply represent residual pinocytic activity accompanied by increased longevity of vesicles, and a maintained accumulation of radiolabel could reflect slow progressive adsorption to the plasma membrane or an increase in adsorption due to cell death over the culture period.

Pinocytosis in the small intestine of suckling mammals has been suggested as a possible mechanism by which maternal antibodies can be transferred to the fetal circulation and it has been observed that this transfer decreases dramatically after a period of time (18-21 days in the rat), the process being termed 'gut closure'. It would seem that uptake of ^{125}I -PVP declines concurrently with failing antibody transfer (Clarke & Hardy, 1969). Steroids have been implicated as the normal physiological controllers of 'closure' in the gut epithelium. Daniels *et al.*, (1973) observed that plasma cortisol concentration was consistently low in rats between 5 and 28 days, whereas there was an increase in the plasma corticosterone level at 18- 21 days. They found that injection of cortisone acetate at 5 days induced precocious closure, and injection of corticosterone produced a transient decrease in ^{125}I -PVP uptake. In the present experiment, using a well defined in vitro system, it was found that corticosterone (12.5 $\mu\text{g}/\text{ml}$) showed some tendency to inhibit the uptake of ^{125}I -PVP after approximately 4-5h of incubation, whereas

cortisone acetate (35 µg/ml) produced no effect. Maintenance of yolk sacs in culture for 24h before addition of radiolabelled substrate and preincubating in the presence and absence of cortisone acetate (results not reported above) did not clarify the position. Although the yolk sacs exposed to cortisone acetate showed a lower rate of ^{125}I -PVP accumulation, so did the untreated yolk sacs and therefore the effects observed could be attributable simply to the culture conditions. To obtain a more informative set of data these preliminary experiments should be expanded and a study of the effect of different concentrations of corticosteroids carried out.

Exocytosis, particularly secretion, is strongly dependent on calcium ions and Douglas (1968) suggested that the role of calcium in secretion was to link a trigger at the plasma membrane with the exocytic event. It is possible that calcium has a similar role in endocytosis and this is discussed at length by Stossel (1977). In these experiments, when the calcium was removed from the culture medium by adding EGTA, uptake of ^{125}I -PVP ceased. Normal capture was completely restored if an equal concentration of CaCl_2 was added with the EGTA, indicating that the absence of calcium was responsible for the effect rather than some pharmacological action of EGTA itself. Additional experiments showed that MgCl_2 does not have the ability to restore uptake, so the requirement for pinocytosis was not simply for a fixed quantity of divalent cation. Hemmaplardh & Morgan, (1977) have shown that calcium is important in regulating and binding of transferrin to reticulocyte receptors and Seglen & Gjessing (1978) showed that attachment of rat hepatocytes to serum coated polystyrene culture dishes requires divalent cations (magnesium being more effective than calcium, but a combination of both stimulated maximum attachment).

Recently Pratten & Lloyd(1979) have shown that the uptake of ^{125}I -PVP by rat peritoneal macrophages is severely inhibited on removal of exogenous calcium, but again the process is restored by adding calcium back to the system. Pinocytosis in Amoeba proteus was found to be inhibited by 0.5mM calcium (Josefsson et al., 1975) and it was proposed that in this system calcium is functional in the control of pinocytic channel formation. It was shown that translocation of calcium from membrane sites into the cell occurred during this process.

Theophylline inhibits phosphodiesterase activity and causes an elevation in the intracellular level of cAMP. In the rat visceral yolk sac it caused a concentration -dependent inhibition of the uptake of ^{125}I -PVP, and at 180 $\mu\text{g}/\text{ml}$ caused 25% inhibition. This result contrasts with the report of Brown and Segal (1978) that theophylline at this concentration did not affect the uptake of yeast invertase by the rat visceral yolk sac. However, these authors did find that dibutyl cAMP itself was inhibitory and theophylline augmented the inhibitory effect of epinephrine. There are many other reports on the literature that describe cAMP inhibition and these are reviewed in Section 1.1.1. The study of pinocytosis of ^{125}I -PVP in rat peritoneal macrophages by Pratten & Lloyd (1979) showed that dibutyl cAMP and theophylline were slightly stimulatory at low concentrations(1 $\mu\text{g}/\text{ml}$ and 10 $\mu\text{g}/\text{ml}$ respectively) and inhibitory at higher concentrations.

The electrophysiology of the cell membrane may play an important role in the control of pinosome formation, and it has been shown that membrane depolarization accompanies endocytic stimulation in Amoeba proteus (Josefsson et al., 1975) and alveolar macrophages (Gormley et al., 1978).

Ouabain inhibits sodium/potassium flux across the plasma membrane and thus provides the possibility to disrupt the normal membrane potential difference. Ouabain had a biphasic effect on the uptake of ^{125}I -PVP in the rat visceral yolk sac with marginal stimulation at low concentration ($10\mu\text{g/ml}$) and marked inhibition at higher concentration ($100\mu\text{g/ml}$). Again these experiments are by no means extensive, but perhaps one or two tentative explanations may be suggested. Possibly a small depolarization is a trigger for endocytosis, explaining why more pinocytosis occurred at $10\mu\text{g/ml}$, but when the distribution of sodium and potassium is more seriously disrupted it becomes much more difficult to initiate membrane infolding. Naccache et al., (1977) showed that stimulation of lysosomal enzyme secretion from rabbit polymorphonuclear leucocytes was accompanied by influx of $^{22}\text{Na}^+$ and efflux $^{42}\text{K}^+$ as well as displaying $^{45}\text{Ca}^{2+}$ influx and elevated intracellular Ca^{2+} levels.

To summarize, several important properties of the pinocytic mechanism in the rat yolk sac have been elucidated by this study. The major points of significance are as follows:-

1. Pinocytic uptake of ^{125}I -PVP, ^{125}I -dBSA and colloidal ^{198}Au is completely inhibitable by low temperature and the presence of metabolic inhibitors.
2. Pinocytic uptake of ^{125}I -PVP is partially sensitive to cytoskeletal inhibitors.

This information, coupled with our previous knowledge of yolk sac morphology and the pinosome size distribution observed within the active cells, allows speculation about the nature of the pinocytic mechanisms in operation. A system that completely

prevents pinocytic uptake (including a metabolic inhibitor or at low temperature) has great potential for the study of interaction of substrates with the cell surface. It can be seen here that the quantity of ^{125}I -PVP, ^{125}I -dBSA and colloidal ^{198}Au associated with the tissue under these conditions is directly proportional to the quantity of binding of these substrates, as postulated by their rates of pinocytic capture. Brief cessation of pinosome formation could also be useful during treatment of the cell surface e.g. digestion of specific binding sites by enzymes, as this would stop the chemical modifier penetrating the cell and thus avoid intracellular damage. After treatment the cells or tissue could be returned to normal conditions allowing the resumption of endocytosis.

Several substances were found to slow down the rate of pinosome formation including corticosterone, theophylline, ouabain and intracellular calcium. Their direct relevance to the control of pinocytosis remains unclear and the failure to discover a substance that enhanced the rate of vesicle formation did not help to clarify the situation. The functional significance of pinocytosis in the visceral yolk sac is not yet fully understood, but perhaps fine control of pinocytic rate is not an important feature of the process in this tissue. It is already known that the system can maintain constant pinocytic uptake for long periods of time, (possibly from 15.5 days until term (Ibbotson, 1978)).

It should be stressed that although most of the conditions and substances used in this Chapter have been reported by other workers as non-detrimental or non-toxic to living cells, it is not known whether the inhibition of pinocytosis is wholly or partially

due to specific effects or as a more general result of cell injury. The recovery experiments undertaken did not help to clarify this position. It would be possible to extend the investigations using the metabolic inhibitors to include the measurement of intracellular ATP levels in the yolk sac after exposure to iodoacetate and 2,4-dinitrophenol and thus determine whether they genuinely interfere with metabolism.

CHAPTER 5.

EFFECT OF POLYAMINO ACIDS AND DEXTRAN DERIVATIVES

ON PINOCYTOSIS IN THE RAT VISCERAL YOLK SAC.

5.1. INTRODUCTION

The rate of endocytosis is modified by many substances, including proteins, peptides, polyamino acids, amino acids, nucleic acids, carbohydrates and even certain inorganic ions. The effects observed may be transient or prolonged, markedly stimulatory or severely inhibitory, and in some cases the response appears to be species specific. Cohn & Parks (1966b) found that pinocytosis in the mouse macrophage could be stimulated by increasing the concentration of newborn calf serum in the culture medium. The observation prompted further study aimed at isolating the active stimulatory components present within the serum; the number of vesicles visible in the macrophage were used as a measure of pinocytic activity. It was found that anionic compounds including polyglutamic acid, dextran sulphate, and albumin and fetuin isolated from the serum were potent stimulators of vesicle formation, whereas cationic substances such as histone, poly-L-lysine and poly-L-ornithine showed no effect.

Findings with other cell types have demonstrated the stimulatory capacity of cationic compounds. Uptake of protein by sarcoma 180 cells is enhanced by polylysine, polyornithine, and DEAE-dextran (Ryser & Hancock, 1965); pinocytosis in amoeba is stimulated by polylysine (Chapman-Andresen, 1971); polyarginine increases the transport of bovine IgG in newborn pig intestine (Smith 1968) and protamine induces pinocytosis in mouse skeletal muscle (Jirmanova et al., 1977).

Recently, reports of polycation stimulation of macrophage endocytosis have appeared in the literature and these reports are difficult to reconcile with the earlier observations of Cohn & Parks

(1966b). Seljelid et al., (1973) showed that poly-L-lysine increases the uptake of [^3H] reovirus double-stranded RNA by mouse macrophages, but they used a radiotracer method for quantitation of endocytosis so perhaps this calls into question the interpretation of the vesicle-counts used by Cohn & Parks (1966b) as a measure of endocytic activity. The observation of Westwood & Longstaff (1976) that polycations enhance the uptake of carbon and benzpyrene particles by rat macrophages is more difficult to explain in this context as they too used morphometric criteria for their analysis. To resolve this latter discrepancy a species difference between rat and mouse peritoneal macrophages must be postulated.

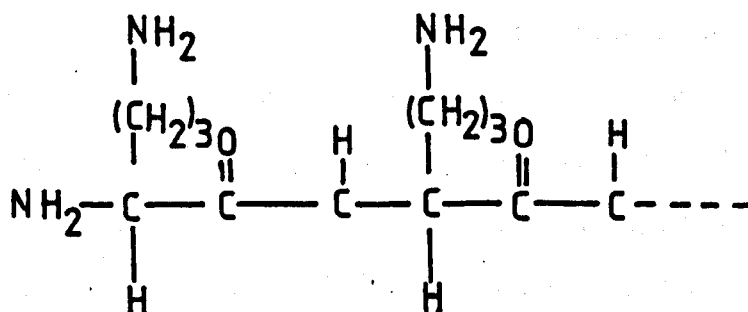
Harmonization of the data in the literature is difficult as several experimental systems have been used, in most cases different substrates employed and the methods used to quantitate pinocytosis vary considerably. In the investigation reported here three polyamino acids and three dextran derivatives have been used in an attempt to define comprehensively the action of the modifiers in the rat visceral yolk sac. Three radiolabelled substrates were used to determine the degree of substrate specificity of any changes that were observed.

Polyamino acids are synthetic homopolymers made from a single amino acid. At neutral pH they assume loose helical coils from which radiate positively or negatively charged groups depending on the particular amino acid used. Poly-L-ornithine (m.wt. 100,000 - 200,000), poly-L-lysine (m.wt. > 70,000), poly-L-glutamic acid (m.wt. 50,000 - 100,000) were chosen for this study as they have been widely used before. Structures and molecular weights are shown in Fig.5.1. Ryser & Hancock (1965) showed that polyamino acids can enter the cell very rapidly by pinocytosis, so the L-isomers were

FIG. 5.1. POLYAMINO ACID STRUCTURE

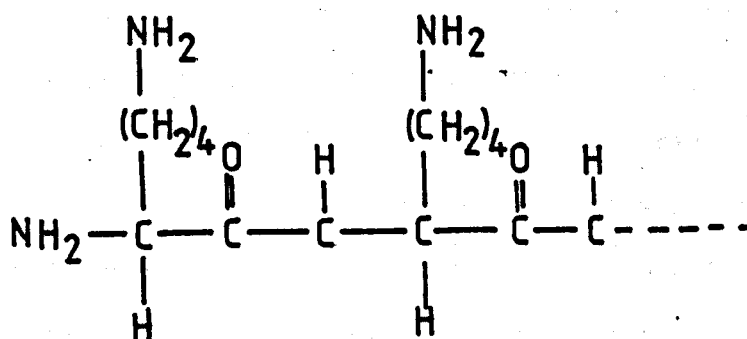
Polyornithine

(m.wt. 100,000 -
200,000)



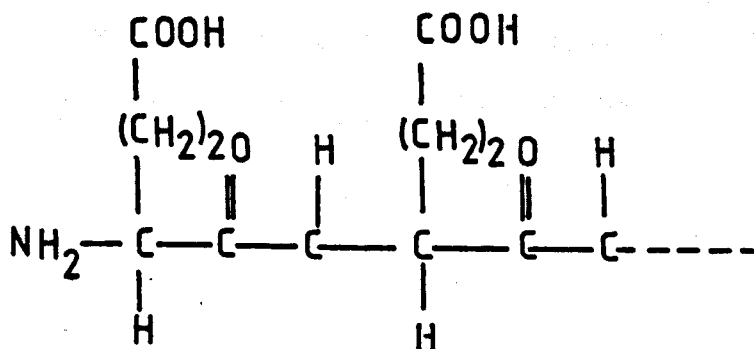
Polylysine

(m.wt. > 70,000)



Polyglutamic acid

(m wt 50,000 -
100,000)



used in the hope that subsequent intracellular degradation would prevent the accumulation of high intracellular levels.

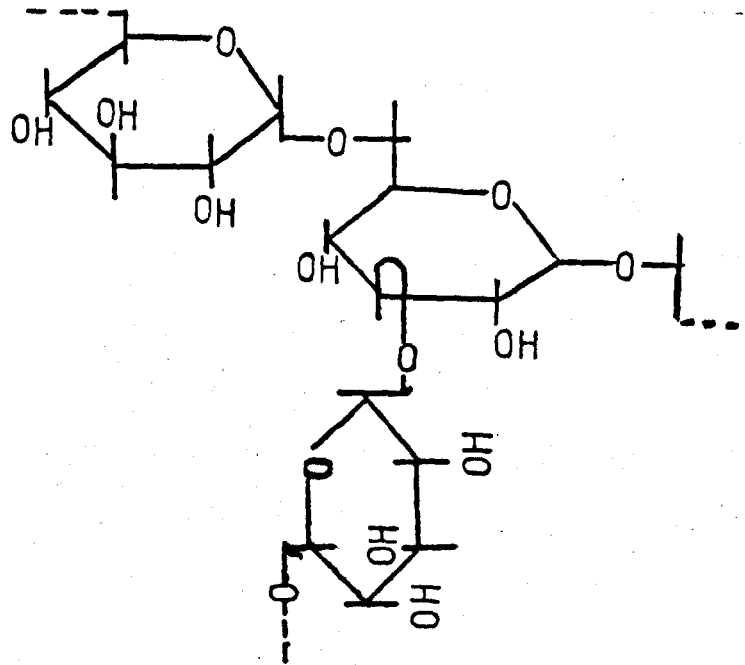
Dextrans are polymers of D-glucopyranose and are synthesised from sucrose by several bacterial species. The majority of the linkages in the chain are (α 1,6) but a low percentage of (α 1,3) bonds gives rise to some branching. In general the molecules assume loose helices at neutral pH. A variety of substituent groups can be introduced via the hydroxyl groups, producing either cationic or anionic derivatives. Dextran (average m.wt.500,000), dextran sulphate (average m.wt.500,000), DEAE-dextran (average m.wt. 500,000) were used, structures and molecular weights being shown in Fig.5.2.

The molecular weights of compounds in both groups were chosen carefully, as it has been shown that the stimulatory capacity of both types of compound is related to their molecular size (Ryser,1967). As far as possible dextrans and polyamino acids of comparable molecular weight ranges were used. The three substrates which were used to quantitate pinocytosis were ^{125}I -PVP, ^{125}I -dBSA and colloidal ^{198}Au . The way in which pharmacological agents modify pinocytosis may be dependent on the mechanism of substrate capture, so it is important to stress here that ^{125}I -PVP was used as a fluid-phase marker, ^{125}I -dBSA was used as an adsorptive marker and colloidal ^{198}Au was used as an interesting intermediate.

FIG. 5.2. STRUCTURE OF DEXTRAN AND DERIVATIVES

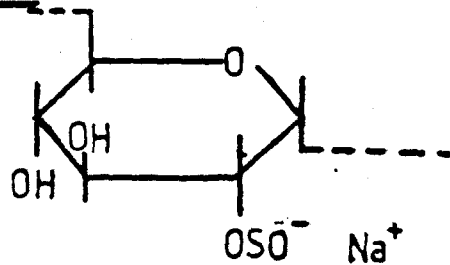
DEXTRAN

(av. m.wt. 500,000)



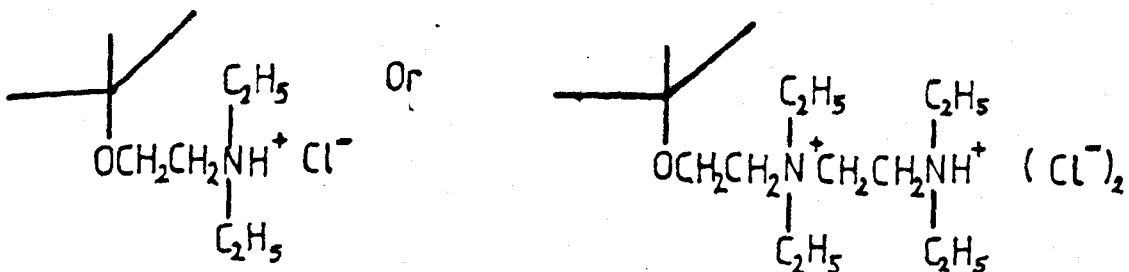
DEXTRAN SULPHATE

(av. m.wt. 500,000)



DEAE - DEXTRAN

(av. m.wt. 500,000)



5.2. RESULTS

Quantitation of substrate capture was carried out as described in Chapter 2. The polyamino acids and dextran derivatives were introduced into the culture flasks together with the substrate. Incubations were generally carried out for 6.5h unless otherwise indicated.

5.2.1. Effect of polyamino acids on the uptake of ^{125}I -PVP ^{125}I -dBSA and colloidal ^{198}Au .

Table 5.1. shows the results obtained when the polyamino acids (5, 10, and 25 $\mu\text{g/ml}$) were added to cultures where ^{125}I -PVP was used as a pinocytotic marker. Uptake was in all cases linear with time and it can be seen that there was no significant change in the Endocytic Index of ^{125}I -PVP except in the case of poly-L-lysine at a concentration of 25 $\mu\text{g/gml}$ which produced approximately 50% inhibition. A polyamino acid concentration of 10 $\mu\text{g/ml}$ was chosen for all further experiments.

The cationic polyamino acids decreased the uptake of ^{125}I -dBSA by approximately 50%, whereas poly-L-glutamic acid caused no significant change. (Table 5.2.). Separation of the net ^{125}I -dBSA uptake into its two components of tissue accumulation and release of catabolized material, indicated that protein degradation was not specifically decreased during cationic inhibition (Fig.5.3).

Poly-glutamic acid caused no change in the rate of uptake of colloidal ^{198}Au , but in this case poly-L-ornithine and poly-L-lysine markedly enhanced tissue accumulation. The stimulation was non-linear with time, thus preventing the calculation of an Endocytic Index, but Fig.5.4. shows a typical experiment in this series.

To investigate the nature of the stimulation produced with

TABLE 5.1. EFFECT OF POLYAMINO ACIDS ON THE UPTAKE OF ^{125}I -PVP

| Polyamino Acid | Concentration ($\mu\text{g/ml}$) | Mean Endocytic Index (\pm S.E.) of ^{125}I -PVP | Mean Percentage of matched Control. |
|----------------------|---------------------------------------|--|---|
| NONE | - | 1.99 ± 0.10 | 100 |
| POLY-L-ORNITHINE | 5 | 2.07 ± 0.12 | 85 |
| | 10 | 1.49 ± 0.14 | 103 |
| | 25 | 1.64 ± 0.08 | 79 |
| POLY-L-LYSINE | 5 | 2.04 ± 0.10 | 90 |
| | 10 | 1.35 ± 0.04 | 81 |
| | 25 | 1.14 ± 0.10 | 54 |
| POLY-L-GLUTAMIC ACID | 5 | 1.85 ± 0.15 | 82 |
| | 10 | 1.68 ± 0.48 | 89 |
| | 25 | 1.67 ± 0.41 | 81 |

TABLE 5.2. EFFECT OF POLYAMINO ACIDS (10 μ g/ml) ON THE UPTAKE
OF 125 I-dBSA AND COLLOIDAL 198 Au.

| Polyamino Acid (10 μ g/ml) | Mean Endocytic Index (\pm SE) of 125 I-dBSA | Mean Endocytic Index (\pm S.E.) of Colloidal 198 Au |
|-----------------------------------|--|---|
| None | 56.44 \pm 4.24 | 3.28 \pm 0.86 |
| POLY-L-ORNITHINE | 26.18 \pm 10.23 (41%) | NON-LINEAR STIMULATION |
| POLY-L-LYSINE | 30.90 \pm 11.24 (48%) | NON-LINEAR STIMULATION |
| POLY-L-GLUTAMIC ACID | 55.76 \pm 8.32 (107%) | 2.04 \pm 0.52 (67%) |

Figures in parentheses are the mean percentage values estimated from each Endocytic Index related to its matched control.

FIG. 5.3. EFFECT OF POLY-L-ORNITHINE (10 μ g/ml) ON THE UPTAKE AND DEGRADATION OF 125 I-dBSA

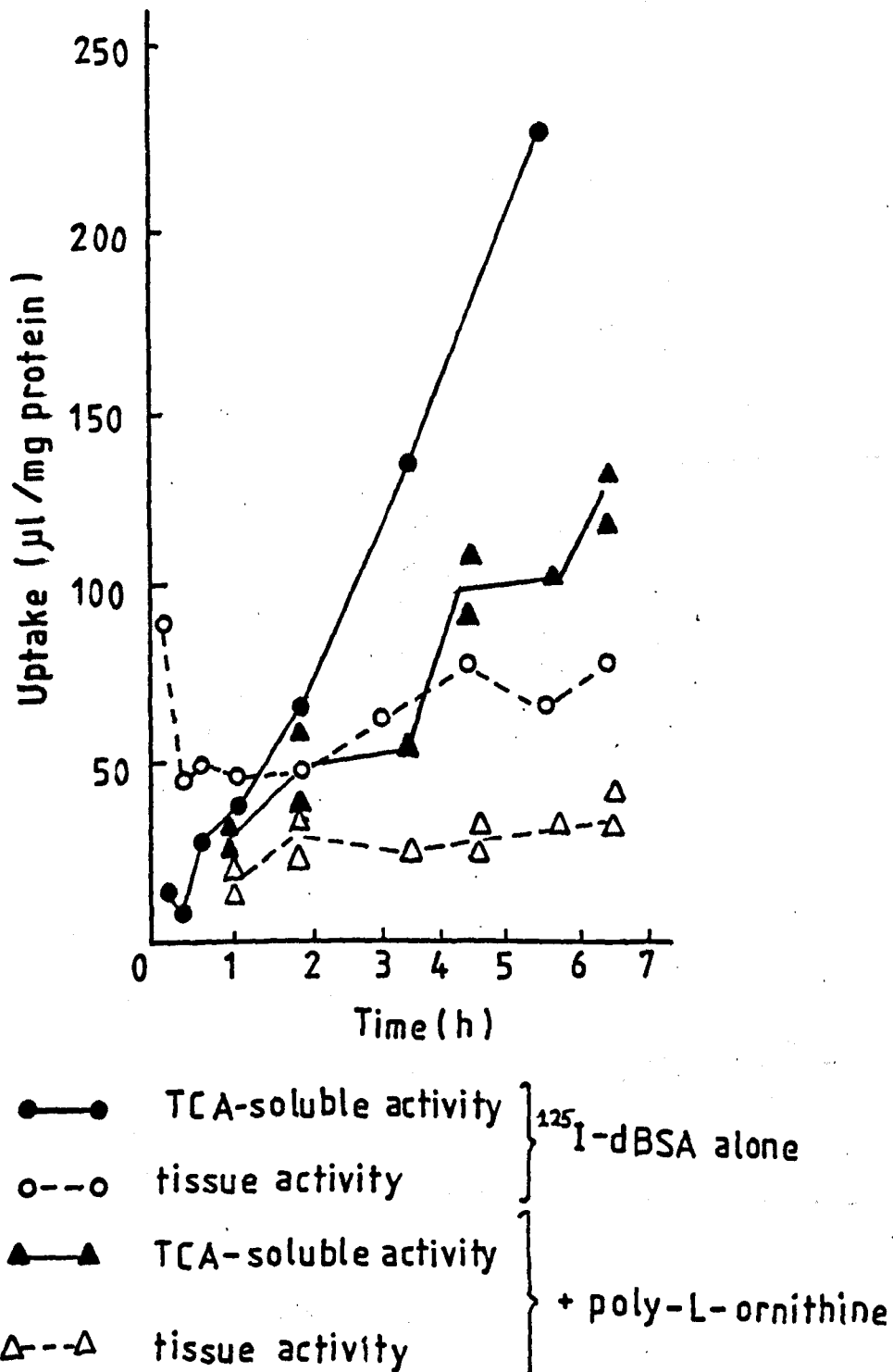
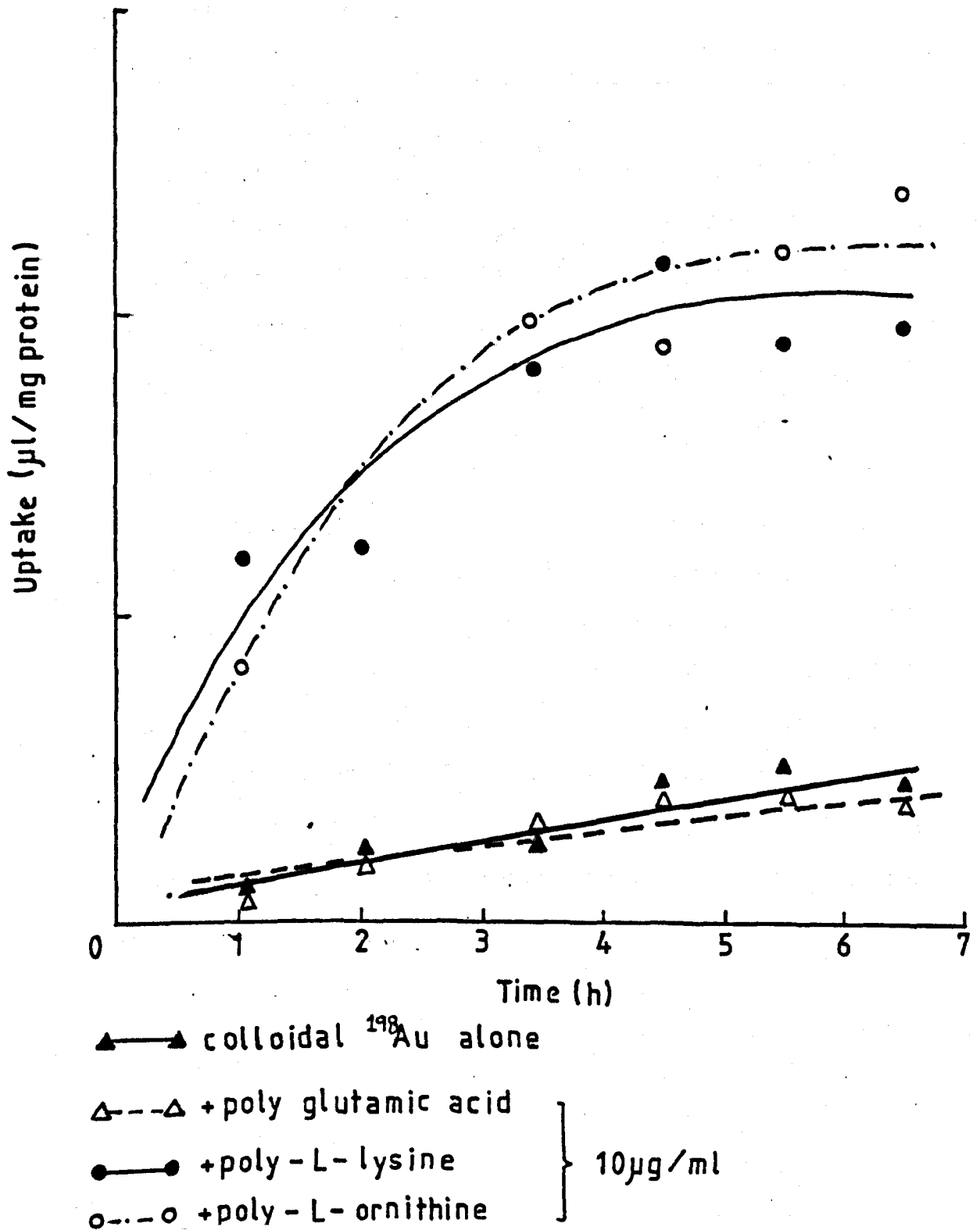


FIG. 5.4. TYPICAL EXPERIMENTS TO SHOW THE EFFECT OF
POLYAMINO ACIDS ON THE UPTAKE OF COLLOIDAL ^{198}Au



colloidal ^{198}Au further experiments were carried out with poly-L-ornithine as a modifier. Fig.5.5. shows the mean stimulation obtained with several batches of colloidal ^{198}Au over an extended time period up to 12h and Fig.5.6. shows the breakdown into mean increase observed with each batch of colloidal ^{198}Au . It can be seen that enhancement is observed with all the batches, but there does appear to be a distinct difference in the level at which accumulation begins to plateau.

5.2.2. Effect of Dextrans on the Uptake of ^{125}I -FVP, ^{125}I -dBSA and Colloidal ^{198}Au .

To test the effect of dextrans on the uptake of ^{125}I -PVP concentrations of 50, 100 and 500 $\mu\text{g/ml}$ were used for each derivative. The results obtained are shown in Table 5.3. Again there was no significant change in the rate of uptake at any concentration except for the charged derivatives at 500 $\mu\text{g/ml}$, which were probably cytotoxic. A concentration of 50 $\mu\text{g/ml}$ was chosen for further experiments.

Table 5.4. shows that none of the dextrans had any effect on the rate of uptake of ^{125}I -dBSA. This directly contrasts with the cationic polyamino acid inhibition described earlier.

The cationic DEAE-dextran enhanced the uptake of colloidal ^{198}Au in a linear fashion by approximately two fold, Table 5.4. Again dextran and dextran sulphate had no effect. A typical experiment in this series is included (Fig.5.7.) so comparison can be made with the stimulation observed with cationic polyamino acids which was non-linear.

FIG. 5.5. POLY-L-ORNITHINE (10 $\mu\text{g}/\text{ml}$) STIMULATION
OF COLLOIDAL ^{198}Au UPTAKE

Mean obtained with four batches of colloidal ^{198}Au

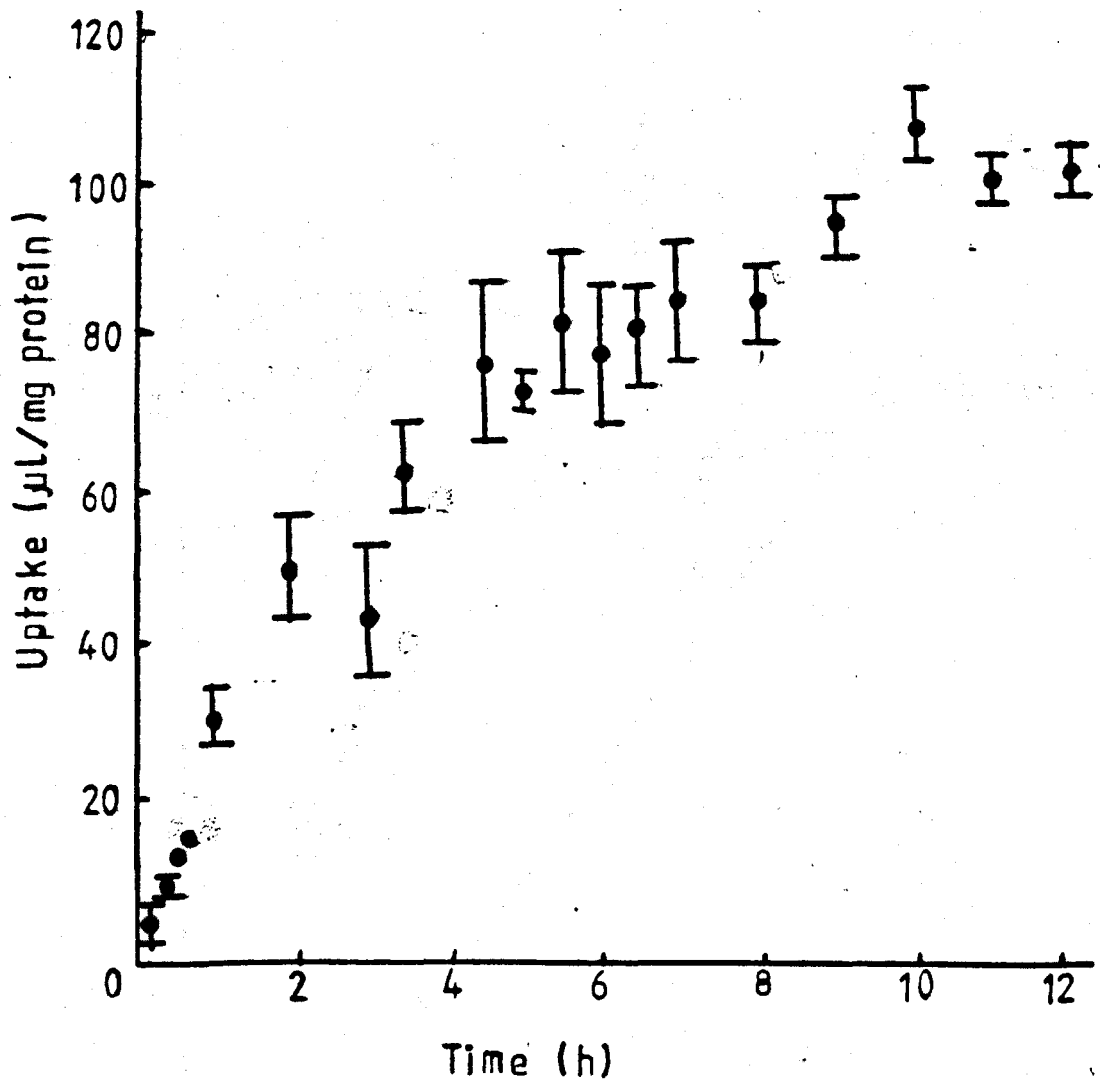


FIG. 5.6. POLYORNITHINE STIMULATION OBSERVED
WITH FIVE BATCHES OF COLLOIDAL ^{198}Au

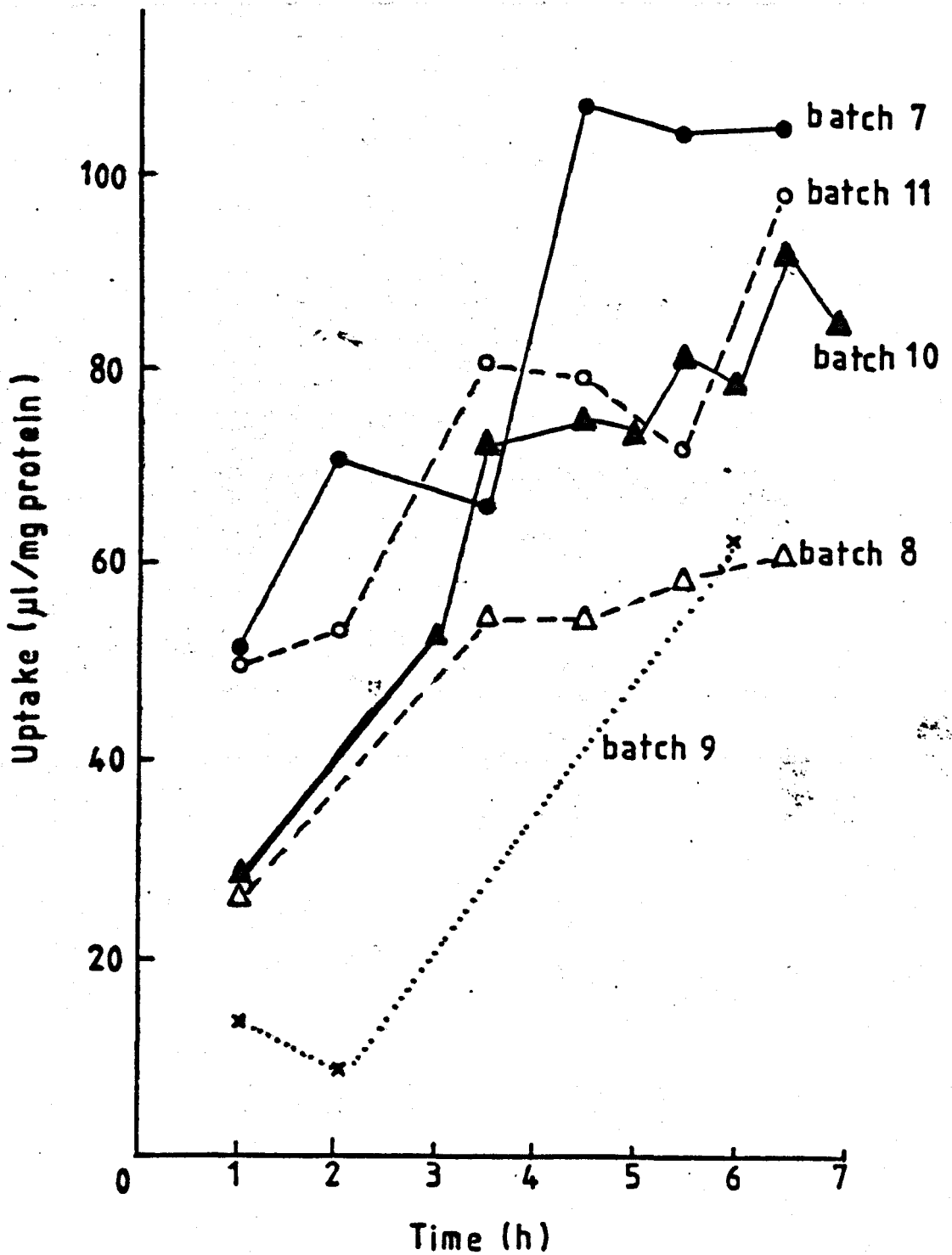


TABLE 5.3. EFFECT OF DEXTRAN AND DEXTRAN DERIVATIVES
ON THE UPTAKE OF ^{125}I -PVP

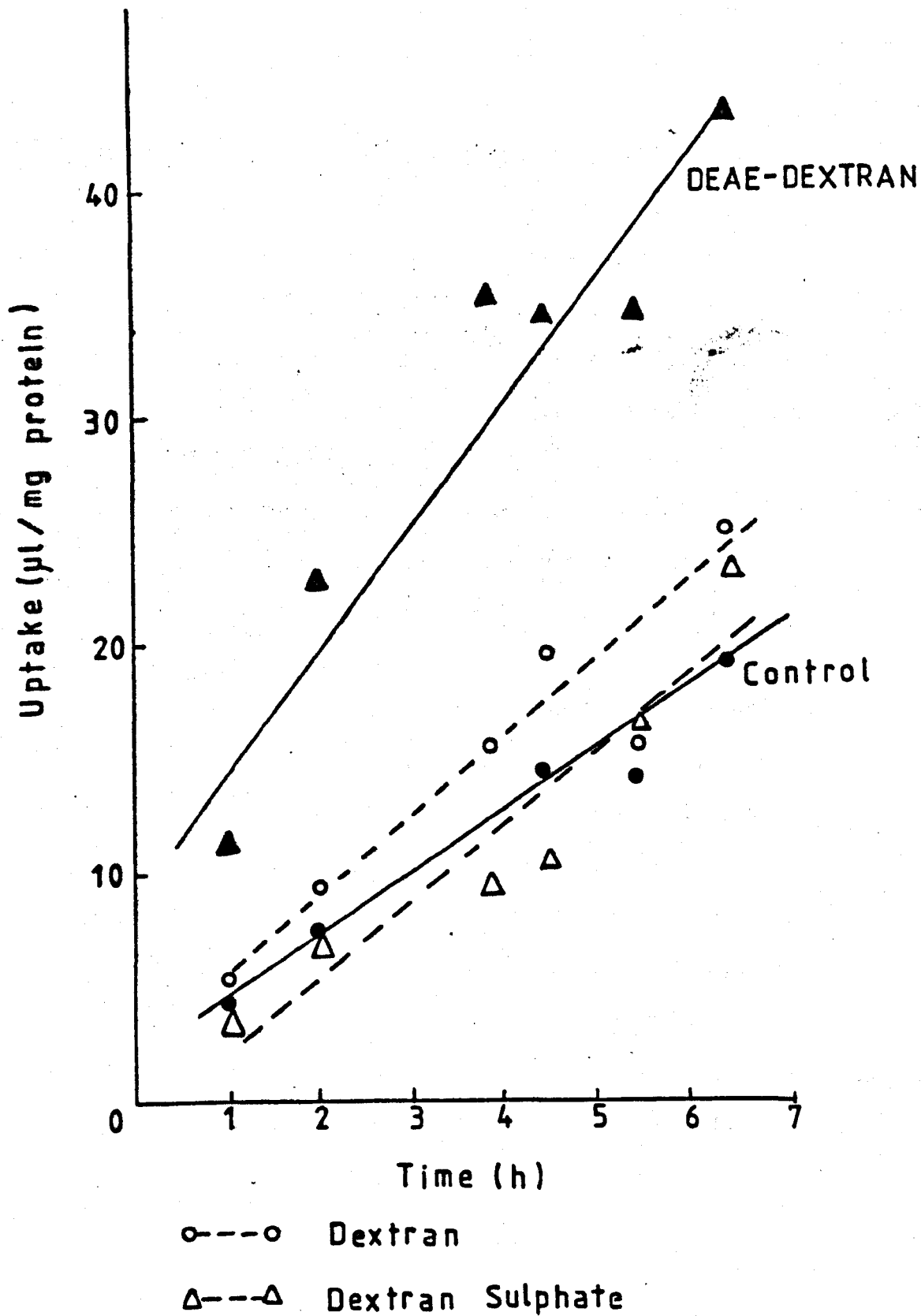
| Dextran | Concentration ($\mu\text{g/ml}$) | Mean Endocytic Index (\pm S.E.) of ^{125}I -PVP | Mean Percentage of Matched Control. |
|---------------------|---------------------------------------|--|--|
| None | - | 1.93 ± 0.12 | 100 |
| DEXTRAN | 50 | 1.68 ± 0.21 | 116 |
| | 100 | 1.53 ± 0.18 | 89 |
| | 500 | 1.73 ± 0.21 | 106 |
| DEAE-DEXTRAN | 50 | 1.91 ± 0.08 | 124 |
| | 100 | 1.77 ± 0.14 | 108 |
| | 500 | 1.22 ± 0.05 | 78 |
| DEXTRAN SULPHATE | 50 | 1.97 ± 0.07 | 89 |
| | 100 | 1.83 ± 0.20 | 82 |
| | 500 | 1.07 ± 0.08 | 41 |

TABLE 5.4. EFFECT OF DEXTRAN AND DEXTRAN DERIVATIVES (50 μ g/ml)
ON THE UPTAKE OF 125 I-dBSA AND COLLOIDAL 198 Au

| Dextran (50 μ g/ml) | Mean Endocytic Index (\pm S.E.) of 125 I-dBSA | Mean Endocytic Index (\pm S.E.) of Colloidal 198 Au |
|----------------------------|--|--|
| NONE | 89.42 \pm 9.76 | 2.68 \pm 0.10 |
| DEXTRAN | 66.76 \pm 7.76 (75%) | 3.63 \pm 0.22 (135%) |
| DEAE-DEXTRAN | 82.60 \pm 9.67 (92%) | 5.18 \pm 0.35 (196%) |
| DEXTRAN SULPHATE | 67.79 \pm 9.39 (75%) | 2.67 \pm 0.33 (100%) |

Figures in parentheses are the mean percentage values estimated from each Endocytic Index related to its matched control.

FIG. 5.7. TYPICAL EXPERIMENTS TO SHOW THE EFFECTS
OF DEXTRAN AND DEXTRAN DERIVATIVES
(50 ug/ml) ON THE UPTAKE OF COLLOIDAL ^{198}Au



5.3. DISCUSSION

Polyamino acids and dextran derivatives exhibit a wide range of effects on pinocytosis in the rat visceral yolk sac, including both stimulation and inhibition. The particular modification observed seemed to be dependent on the substrate used to quantitate pinocytosis as well as the nature of the agent applied. Consistent trends in the data were apparent. Whilst the neutral and polyanionic compounds showed either no effect or inhibition, only the polycationic substances displayed the ability to stimulate uptake.

The cationic poly-L-ornithine and poly-L-lysine altered the control rate of uptake of all three substrates used. The Endocytic Index of ^{125}I -PVP was decreased to 54% of the control level by poly-L-lysine at a concentration of 25 $\mu\text{g}/\text{ml}$. This is perhaps not surprising since both Seljelid et al., (1973) and Ryser & Hancock (1965) showed that poly-L-lysine was toxic at relatively low concentrations (10-25 $\mu\text{g}/\text{ml}$). Although no recovery experiments were undertaken here, it was assumed that cytotoxicity was the likely explanation of these results.

Poly-L-ornithine and poly-L-lysine altered the rates of uptake of ^{125}I -dBSA and colloidal ^{198}Au , but in different ways. Uptake of ^{125}I -dBSA was uniformly decreased over the culture period, the Endocytic Index in the presence of modifier being approximately half the control level; subsequent degradation of the captured ^{125}I -dBSA proceeded normally. In direct contrast colloidal ^{198}Au uptake was greatly enhanced especially during the first 4h of culture, after which the rate of uptake began to decrease and eventually after 6-8h uptake seemed to cease completely. The intrabatch variation in the

actual level of plateau was usually small, but there was considerable difference between batches. It can be seen that batches 8 and 9 achieve an average maximum uptake of approximately 60 $\mu\text{l}/\text{mg}$ protein after 6h, whereas batches 7, 10, and 11, have an uptake of approximately 100 $\mu\text{l}/\text{mg}$ protein at this time. This difference cannot simply be correlated with differences in the control rates of uptake of colloidal ^{198}Au for the relevant batches (see Chapter 4).

Dextran derivatives showed no effect on the uptake of ^{125}I -PVP except at 500 $\mu\text{g}/\text{ml}$ where DEAE-dextran lowered the Endocytic Index to 41% of the control level. Again it is the positively charged polymer which caused the deleterious effect. No effect was observed when dextrans were added to ^{125}I -dBSA cultures, but when colloidal ^{198}Au was used as a pinocytic marker, DEAE-dextran showed a marked stimulation of uptake, which was linear with time, the Endocytic Index being approximately double the control level.

In the rat yolk sac only pinocytosis of colloidal ^{198}Au could be stimulated and a polycation would appear to be a prerequisite for this process. The maximum stimulation relative to the normal rate of colloidal ^{198}Au uptake was observed during the first hour of culture. Uptake values of 30-50 $\mu\text{l}/\text{mg}$ protein were commonplace after 1h in the presence of poly-L-ornithine and poly-L-lysine, and this was still somewhat lower than the forty-fold increases reported by Ryser & Hancock (1965) for poly-L-ornithine enhancement of ^{131}I -HSA uptake into sarcoma-180 cells measured over a 60min period. Ryser & Hancock (1965) also found that the stimulatory capacity of poly-L-ornithine was several times greater than that of poly-L-lysine, but here it is seen that the former is only marginally

more effective than the latter.

In a parallel series of experiments with the rat peritoneal macrophage, polycations were also shown to stimulate pinocytosis (Pratten et al., 1978). Here the same substrates and modifiers were employed and none of the modifiers had any significant effect on the uptake of ^{125}I -PVP. Polyglutamic acid and dextran sulphate inhibited uptake of colloidal ^{198}Au and did not effect the uptake of ^{125}I -dBSA, but poly-L-ornithine, poly-L-lysine and DEAE-dextran all stimulated the uptake of both ^{125}I -dBSA and colloidal ^{198}Au , the stimulated uptake being linear with time. The ability of the macrophage to maintain linear accumulation of colloidal ^{198}Au in the presence of cationic polyamino acids contrasts directly with the transient nature of the stimulation in the yolk sac. The reasons for this difference are not readily apparent at this stage, but an explanation may be postulated based on data reported in the following Chapter (see Section 7.3.). The investigations carried out in the rat peritoneal macrophage are particularly interesting as the results obtained are directly comparable to those reported here for the visceral yolk sac system and taken together the data represent an important comparative study of two pinocytic systems (Pratten et al., 1978). There are several mechanisms by which polycations might modify pinocytic rate, that can be summarized as follows:-

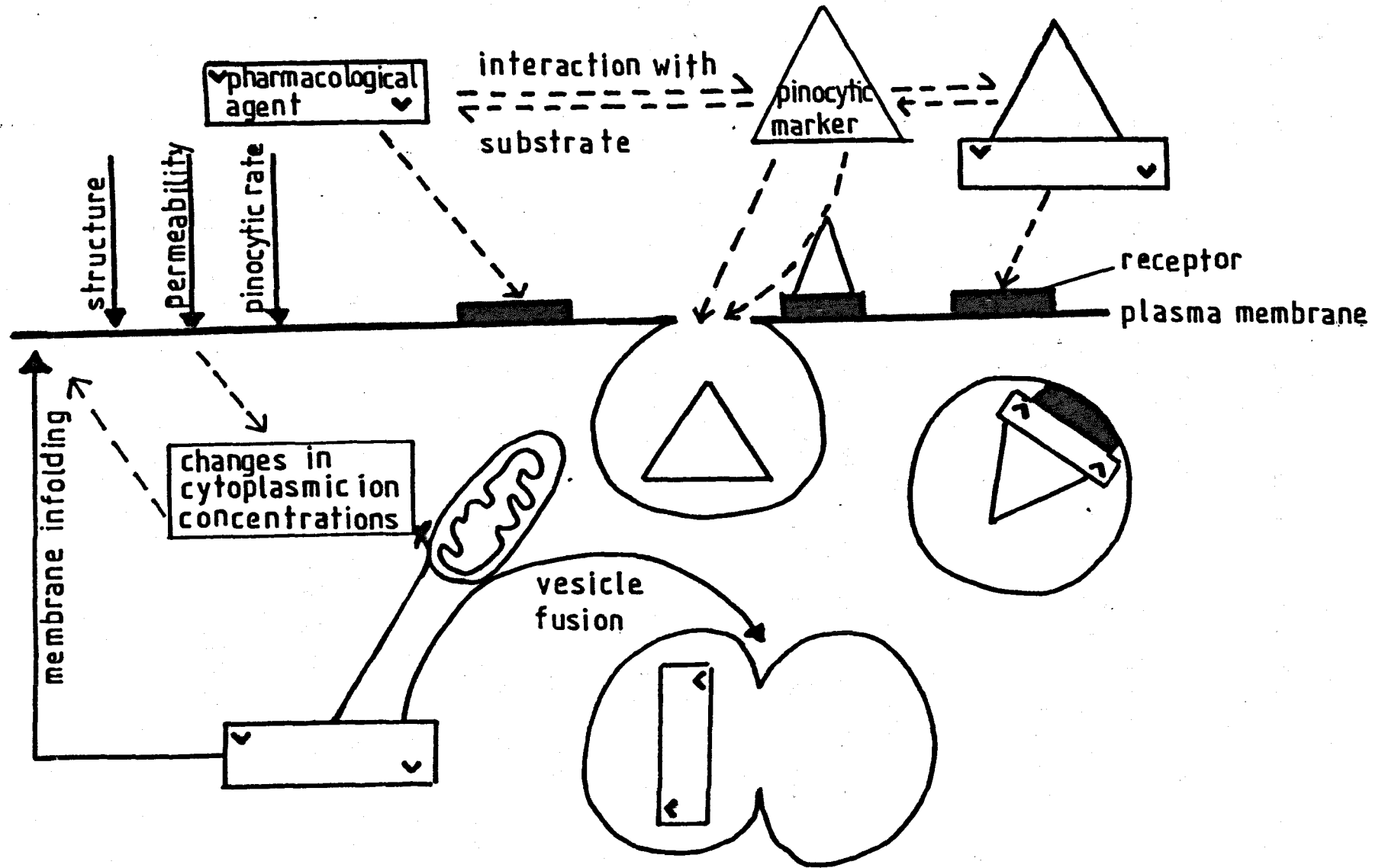
- i) An increase in the rate of pinosome formation.
- ii) Initiation of a 'new' mechanism of pinocytosis either more or less dependent on a microtubule/microfilament system and a metabolic energy supply.
- iii) An alteration in the membrane affinity for the substrate, so that either more or less binding occurs.

Fig. 5.8. is a diagrammatic representation of these three possibilities and shows some of the ways that a pharmacological agent might in theory interact with the system to bring about the change.

Hewitt (1977) explained why polyvalent ligands might be expected to induce membrane curvature and hence enhance the rate of formation of pinocytic vesicles, and recently it has been shown experimentally that the interaction between poly-L-lysine and model membranes containing charged lipids causes charge -induced domain formation which results in changes in surface curvature (Hartman & Galla, 1978). On the basis of this information one might expect at least some of the modifiers employed here to alter the rate of pinosome formation in the visceral yolk sac but none of the substances tested had any effect on the rate of uptake of the fluid-phase marker ^{125}I -PVP except at high concentrations where non-specific toxicity was beginning to take effect. Assuming that the changes in the rate of ^{125}I -dBSA and colloidal ^{198}Au uptake were not due to alterations in the rate of pinosome formation, either of the latter two mechanisms mentioned above may have been in operation.

^{125}I -dBSA and colloidal ^{198}Au enter in both fluid phase and to a greater or lesser extent, by membrane binding, so consequently one might predict that their uptake may be susceptible to modification by substances that could exert an effect on substrate-receptor affinities. The effect of cationic polyamino -acids on the uptake of colloidal ^{198}Au is particularly relevant, as it closely parallels the type of binding curve familiar from classical binding kinetics. Evidence described in Chapter 6 suggests that changes initiated by poly-L-ornithine, poly-L-lysine and DEAE-dextran in conjunction with colloidal ^{198}Au might simply be due to increased membrane

FIG. 5.8. SOME OF THE THEORETICAL ACTIONS OF A PINOCYTIC MODIFIER



binding. The decrease in ^{125}I -dBSA uptake by poly-L-ornithine and poly-L-lysine could also be satisfactorily explained, if there is competition between the polyamino acids and ^{125}I -dBSA for surface receptor sites. It has been shown by Ryser (1965) that polyamino acids enter sarcoma-180 cells much faster than ^{131}I -HSA, which indicates a very high level of surface adsorption. Although the uptake of the modifiers themselves has not been quantitated here, there is no reason to suppose that a similar high rate of internalization does not occur in the visceral yolk sac, and that this might be at the expense of some ^{125}I -dBSA incorporation. Competition between other proteins during pinocytic uptake into the yolk sac has been well documented (Ibbotson, 1978; Livesey, 1979). DEAE-dextran does not change the rate of ^{125}I -dBSA uptake and this would fit into a competition model, as it is unlikely that a carbohydrate molecule could be an efficient competitor in such a situation.

To summarize, on addition of synthetic polyelectrolytes several different effects have been observed in the visceral yolk sac; inhibition of ^{125}I -dBSA uptake in the presence of poly-L-ornithine and poly-L-lysine which was linear with time; stimulation of colloidal ^{198}Au uptake on addition of DEAE-dextran which was linear with time and finally the transient stimulation of colloidal ^{198}Au uptake by poly-L-ornithine and poly-L-lysine.

In Chapter 6 the possible mechanism of pinocytic modification in the rat yolk sac is investigated further.

CHAPTER 6.

THE MECHANISM OF PINOCYTIC MODIFICATION BY CATIONIC POLYMERS.

6.1. INTRODUCTION

Polycations initiate a wide variety of physiological responses in many cell types. They can change the agglutinability of cells (Katalsky, et al., 1959), induce an increase on the area of contact of cells (Bases et al., 1973) alter membrane permeability and morphology (Mamelak et al., 1969) and also stimulate endocytic uptake (see Section 5.1.). It was shown in the previous Chapter that polyamino acids and dextran derivatives modify the pinocytosis of certain substances by the rat visceral yolk sac. Poly-L-Lysine and poly-L-ornithine enhanced colloidal ^{198}Au accumulation for short periods of time but inhibited the uptake of ^{125}I -dBSA, whereas DEAE-dextran stimulated colloidal ^{198}Au uptake for up to 6h. Inability of all the polymers to significantly change the rate of capture of ^{125}I -PVP indicated that they do not change the basal rate of vesicle formation (discussed further in Section 5.3.).

The mechanism of polycation stimulation of pinocytosis is still unclear. It has been suggested that enhancement is achieved by either increased binding of the captured molecules to the plasma membrane (Seljelid et al., 1973) or alternatively by the initiation of a different endocytic mechanism, (Ryser & Hancock, 1965). Here a wide variety of methods were used to investigate the mechanism of polycation modification of pinocytosis in the rat visceral yolk sac. Owing to the large number of polymers and substrates used in the initial study, it was not possible to perform all the experiments with each combination of modifier and substrate. For the most part poly-L-ornithine and DEAE-dextran were used as modifiers and colloidal ^{198}Au was used as a substrate.

The mechanism was investigated by performing experiments to determine the following:-

1. The stereospecificity of polycation stimulation.
2. The metabolic characteristics of stimulated uptake.
3. The effect of stimulating colloidal ^{198}Au capture on the uptake of ^{125}I -PVP.
4. The effect of removal of calf serum on the stimulatory capacity of polycations.
5. The exocytosis of substrate following stimulated uptake.
6. The effect of polycations on colloidal ^{198}Au particle size in the culture medium.

6.2. RESULTS

The quantitation of substrate capture and exocytosis was carried out as described in Chapter 2. Polyamino acids and dextran derivatives were introduced as described in Chapter 5 and the methods used for temperature and metabolically inhibited culture were identical to these described in Chapter 3. Again incubations were carried out for periods up to 7h and exocytosis was measured over a 3h time period.

Incubations carried out in the absence of calf serum were performed using the standard no-serum technique described in Section 2.2. Methodology for sucrose density gradient centrifugation is described in Section 2.9.

For the dual-substrate experiments ^{125}I -PVP and colloidal ^{198}Au were added simultaneously and the incubation was carried out in the usual way. It was possible to quantitate uptake of colloidal ^{198}Au immediately as there is no significant spill-over of ^{125}I emission into the ^{198}Au channel on the gamma counter. Samples were then stored for several weeks until the colloidal ^{198}Au had no measurable activity and then recounted in the ^{125}I channel in order to quantitate uptake of ^{125}I -PVP.

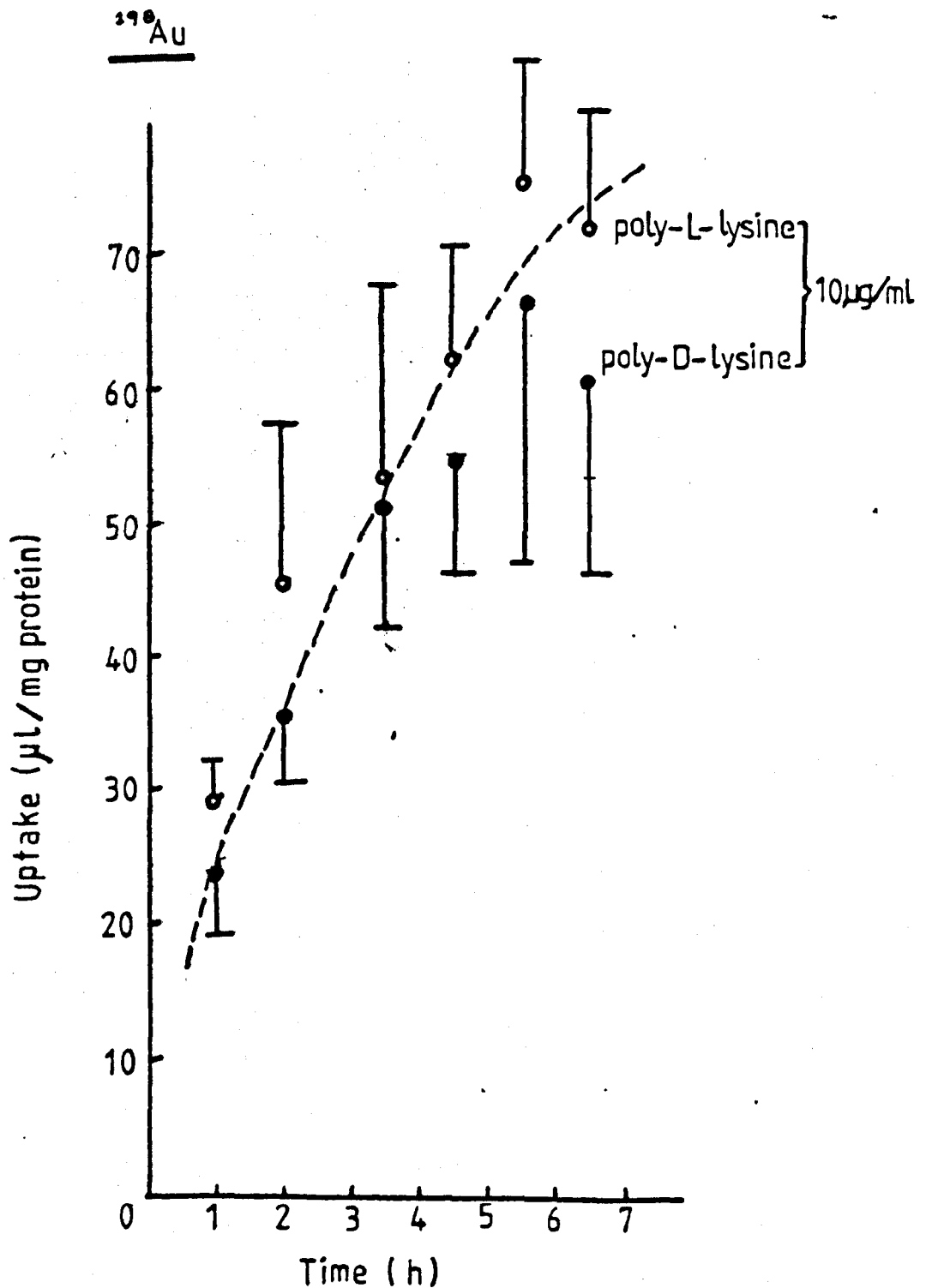
6.2.1. Effect of Poly-L-lysine on the uptake of colloidal ^{198}Au

Poly-D-lysine (10 $\mu\text{g}/\text{ml}$) stimulated the uptake of colloidal ^{198}Au (Fig. 6.1.) to the same extent as poly-L-lysine (see Chapter 5)

6.2.2. Dual-substrate label experiments to determine whether polyamino acid in combination with colloidal ^{198}Au can stimulate fluid incorporation

This experiment enabled the estimation of colloidal ^{198}Au and

FIG. 6.1. EFFECT OF THE DIFFERENT ISOMERS OF
POLY-LYSINE ON THE UPTAKE OF COLLOIDAL



^{125}I -PVP capture simultaneously by the same visceral yolk sacs.

Fig. 6.2. shows the mean uptake of three such experiments carried out without any additions to the culture medium and also in the presence of poly-L-ornithine (10 $\mu\text{g}/\text{ml}$). The basal rates of uptake of the two substrates were not significantly different from those described previously, indicating that neither compound affects the rate of uptake of the other. It can be seen that under stimulated conditions the enhanced colloidal ^{198}Au accumulation did not change the rate of ^{125}I -PVP uptake.

6.2.3. Effect of Metabolic Inhibitors and Low Temperature on the Stimulation of Colloidal ^{198}Au Uptake

Fig. 6.3. shows that DEAE-dextran stimulated colloidal ^{198}Au uptake even in the presence of 50 $\mu\text{g}/\text{ml}$ 2,4-dinitrophenol, a concentration of inhibitor which was sufficient to block pinocytosis of colloidal ^{198}Au almost completely in the absence of stimulator (see Chapter 4). The uptake was linear with time over the culture period and equivalent to the stimulated uptake observed in the absence of 2,4-dinitrophenol. DEAE-dextran-enhanced uptake was less at 3 $^{\circ}\text{C}$ than at 37 $^{\circ}\text{C}$, but retained linearity with time and was considerably higher than colloidal ^{198}Au uptake at 3 $^{\circ}\text{C}$ is virtually negligible (see Chapter 4).

Similarly 2,4-dinitrophenol (50 $\mu\text{g}/\text{ml}$) did not affect poly-L-ornithine stimulation (Fig. 6.4.). At 3 $^{\circ}\text{C}$ poly-L-ornithine facilitated the continuous accumulation of substrate, which was linear over the culture period (Endocytic Index of 4.38 $\mu\text{l}/\text{mg}$ protein/h). This rate of capture at 3 $^{\circ}\text{C}$ was not far removed from the control Endocytic Index of colloidal ^{198}Au measured at 37 $^{\circ}\text{C}$.

To test whether the resistance of poly-L-ornithine stimulation

FIG. 6.2. DUAL SUBSTRATE EXPERIMENT TO ASSESS THE
EFFECT OF POLY-L-ORNITHINE (10 $\mu\text{g}/\text{ml}$) AND
COLLOIDAL ^{198}Au ON THE UPTAKE OF ^{125}I -PVP

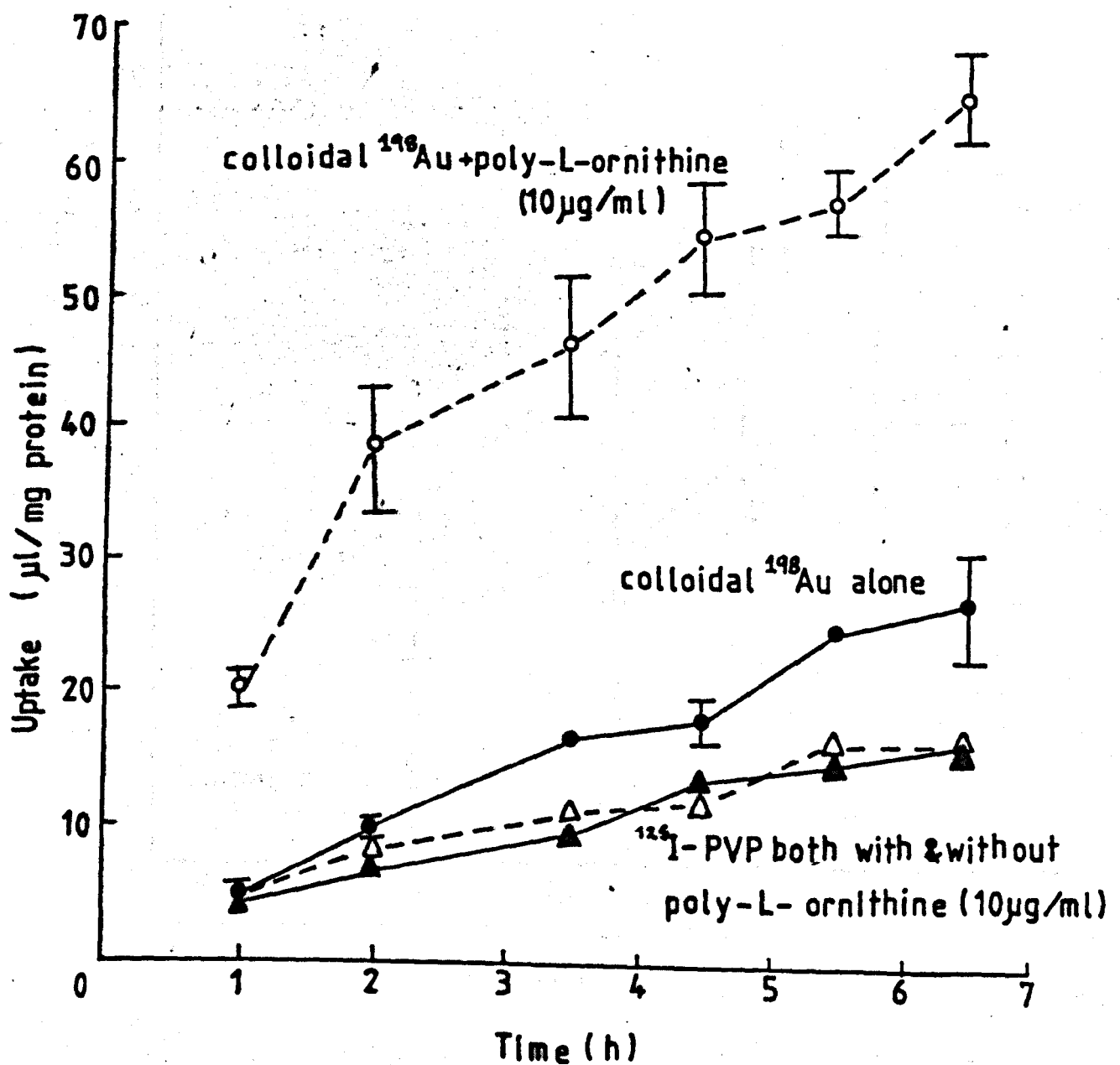


FIG. 6.3. EFFECT OF PINOCYTIC INHITORS ON DEAE-

DEXTRAN STIMULATION OF COLLOIDAL

^{198}Au UPTAKE

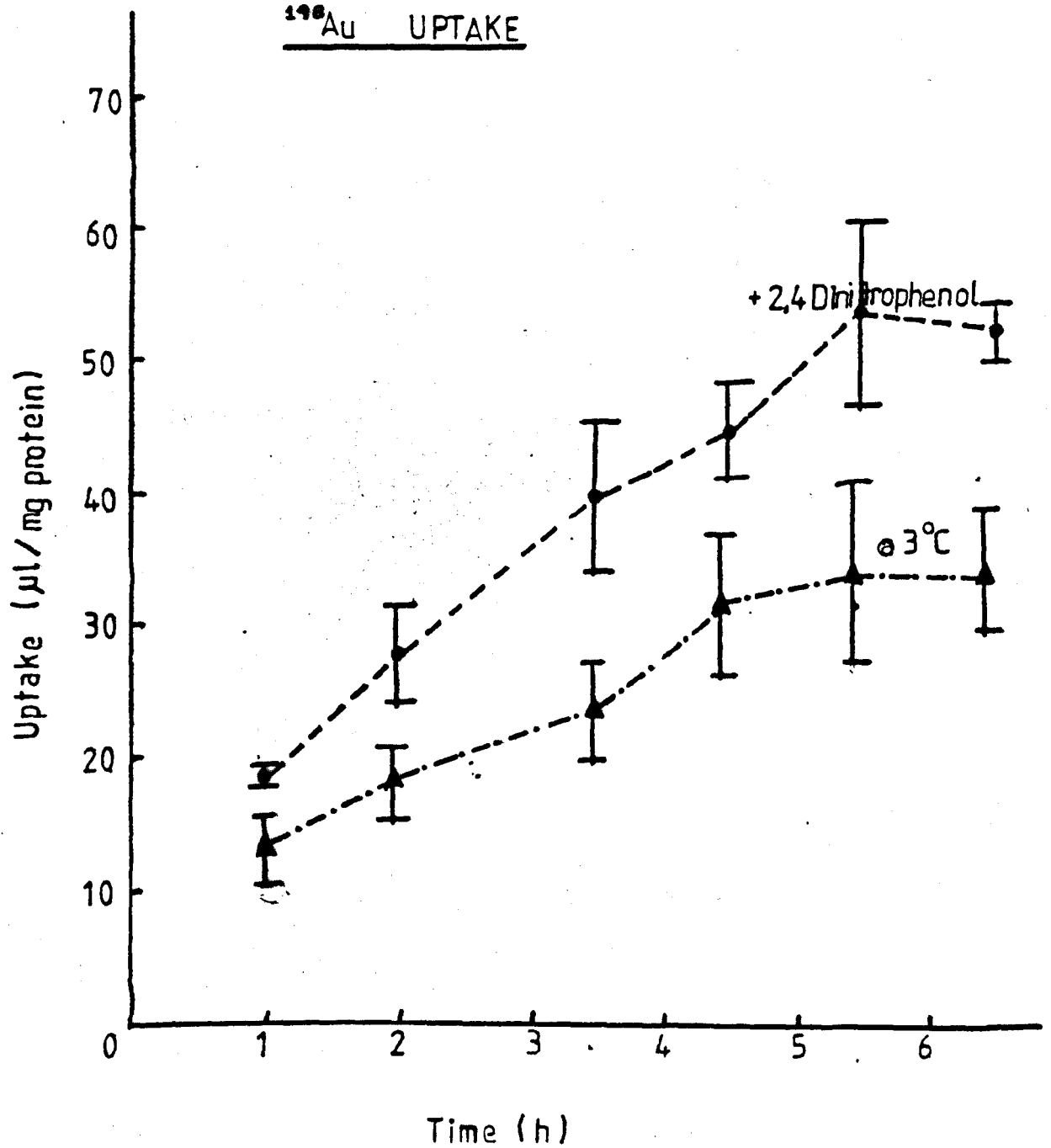
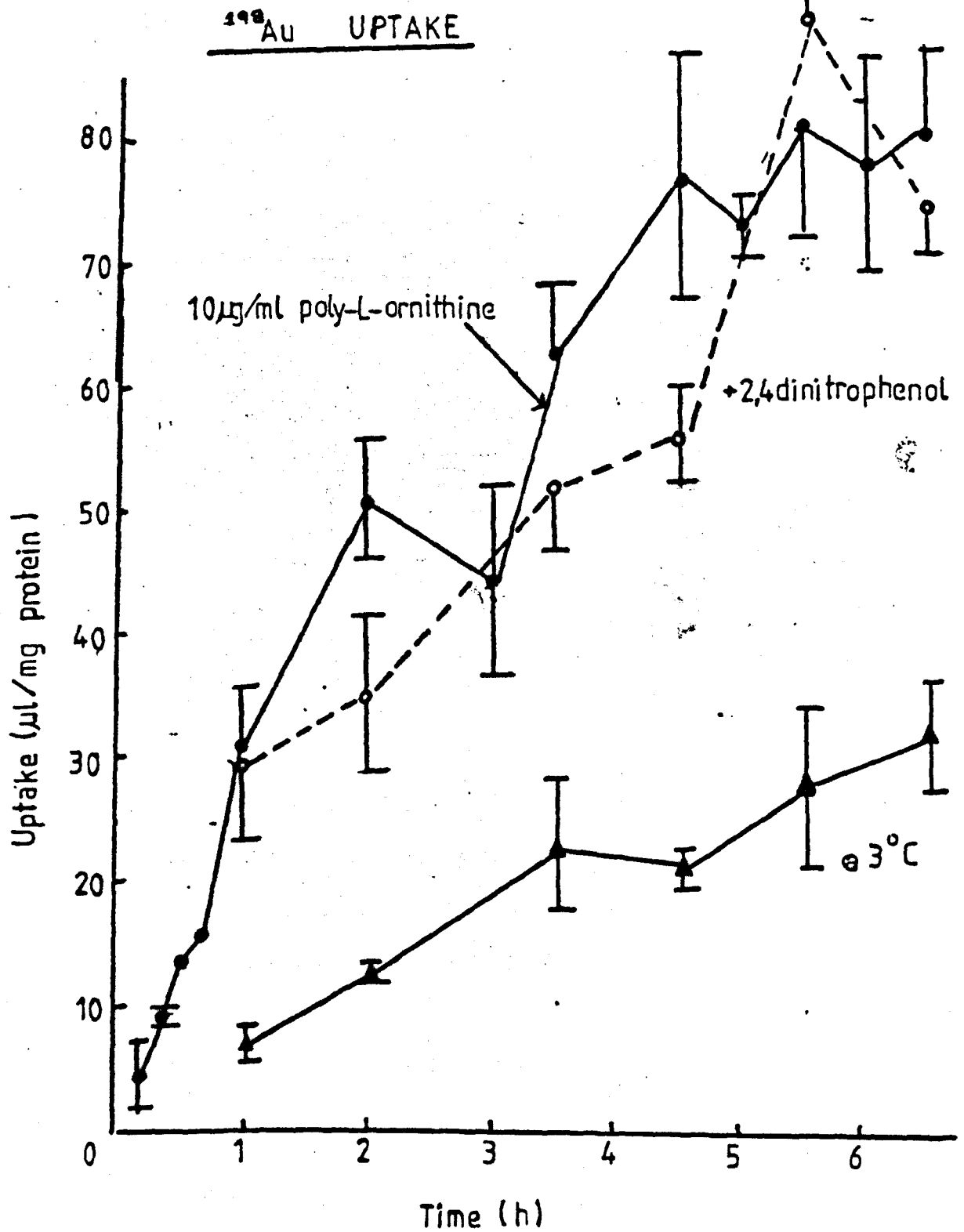


FIG. 6.4. EFFECT OF PINOCYTIC INHIBITORS ON

POLY-L-ORNITHINE STIMULATION OF COLLOIDAL

¹⁹⁹Au UPTAKE



to metabolic inhibition was real effect or simply an artefact caused by the neutralization of 2,4-dinitrophenol inhibition by the addition of poly-L-ornithine, a dual substrate experiment was undertaken, the incubation media containing either:-

- 1) Colloidal ^{198}Au and ^{125}I -PVP
- or
- 2) Colloidal ^{198}Au , ^{125}I -PVP, poly-L-ornithine ($10\text{ }\mu\text{g/ml}$) and 2,4-dinitrophenol ($50\text{ }\mu\text{g/ml}$)

The results are shown in Fig. 6.5. and it can be seen that poly-L-ornithine stimulation of colloidal ^{198}Au uptake in the presence of 2,4-dinitrophenol continued in the same yolk sacs where the uptake of ^{125}I -PVP was inhibited.

6.2.4. Effect of Polyamino Acids on the Uptake of ^{125}I -PVP, ^{125}I -dBSA and Colloidal ^{198}Au in the Absence of Calf Serum

Absence of calf serum from the culture system made little difference to the effect of polyamino acids on ^{125}I -PVP uptake. Both poly-L-ornithine and poly-L-lysine were slightly inhibitory without serum, whereas poly-L-glutamic acid slightly elevated the rate of ^{125}I -PVP uptake. (Fig. 6.6). These changes were small and comparison of the data with their matched controls showed that the differences were not significant. Comparison of modifier-containing cultures, both with, and without serum, showed that absence of calf serum did not significantly change the effect of any polyamino acid on ^{125}I -PVP uptake. (Results are expressed as a percentage of matched controls throughout this section, as the Endocytic Index for ^{125}I -PVP is always slightly higher in the absence of calf serum).

Although one may normally culture yolk sacs with colloidal ^{198}Au in the absence of serum with reasonable success (Ibbotson & Williams, 1979)

FIG. 6.5. SIMULTANEOUS UPTAKE OF COLLOIDAL ^{198}Au
AND ^{125}I -PVP BY YOLK SACS INCUBATED IN
THE PRESENCE OF 2,4-DINITROPHENOL(50 $\mu\text{g}/\text{ml}$)
AND POLY-L- ORNITHINE (10 $\mu\text{g}/\text{ml}$)

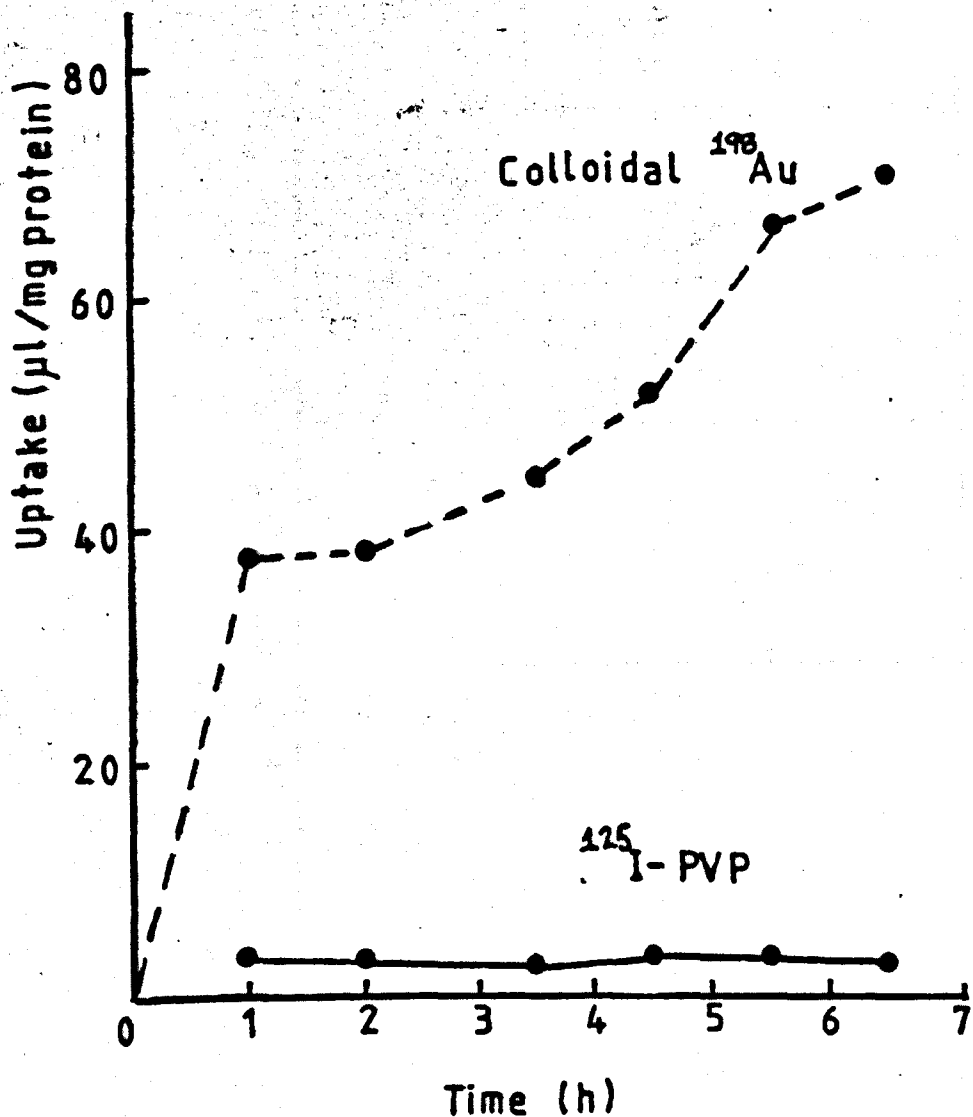
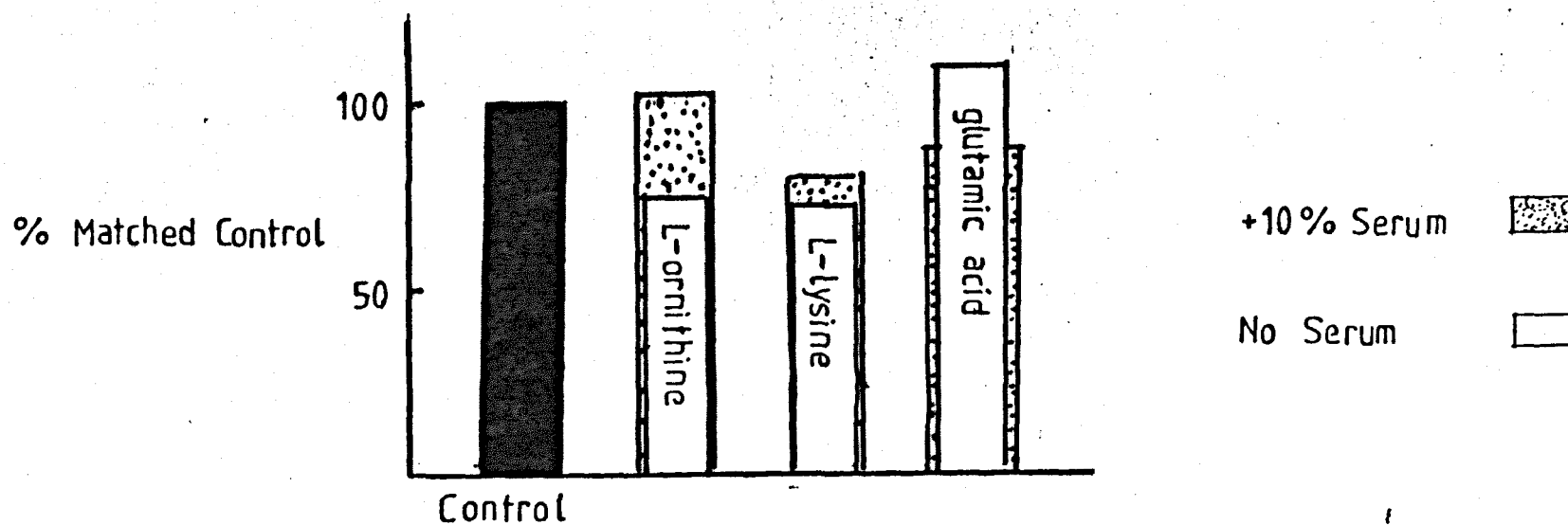


FIG. 6.6. EFFECT OF POLYAMINO ACIDS ON THE UPTAKE OF ^{125}I -PVP



problems were encountered when polyamino acids were introduced into this serum-free system. These are highlighted in Fig. 6.7. The introduction of poly-L-ornithine ($10\text{ }\mu\text{g/ml}$) into a culture flask containing 10ml of TC 199 and colloidal ^{198}Au ($1\text{ }\mu\text{g/ml}$), but no yolk sac caused considerable rapid depletion of colloidal ^{198}Au radioactivity from the medium. It is assumed that the loss reflected poly-L-ornithine facilitated binding of colloidal ^{198}Au to the glass flask. No measurable depletion in the medium was seen in flasks which contained both poly-L-ornithine ($10\text{ }\mu\text{g/ml}$) and 10% calf serum.

Obviously the complication introduced by an unstable medium count made the system less than ideal for estimating the quantity of colloidal ^{198}Au accumulated in the presence of modifier and absence of calf serum. Preliminary results showed that very large quantities of substrate became associated with the tissue so rapidly that the tissue level was at a maximum after 1h. This effect was ofcourse exaggerated by the unrealistically low medium count. It might be possible to devise a method for quantifying substrate capture under these conditions by integrating a medium depletion term into the calculation but this would be quite complicated.

6.2.5. Exocytosis of Colloidal ^{198}Au in the Presence of Poly-L-Ornithine.

The exocytosis of colloidal ^{198}Au over a 3h period is shown in Fig. 6.8. Release of ^{198}Au was $1.55 \pm 0.18\%/h$ under control conditions, whereas the rate of release rose to $5.83 \pm 1.02\%/h$ when $10\text{ }\mu\text{g/ml}$ of poly-L-ornithine was added to culture medium during both the incubation and release phases. This showed that release from the stimulated tissue was some 4 times greater than normal exocytic release.

FIG. 6.7. EFFECT OF MEDIUM COMPOSITION ON COLLOIDAL

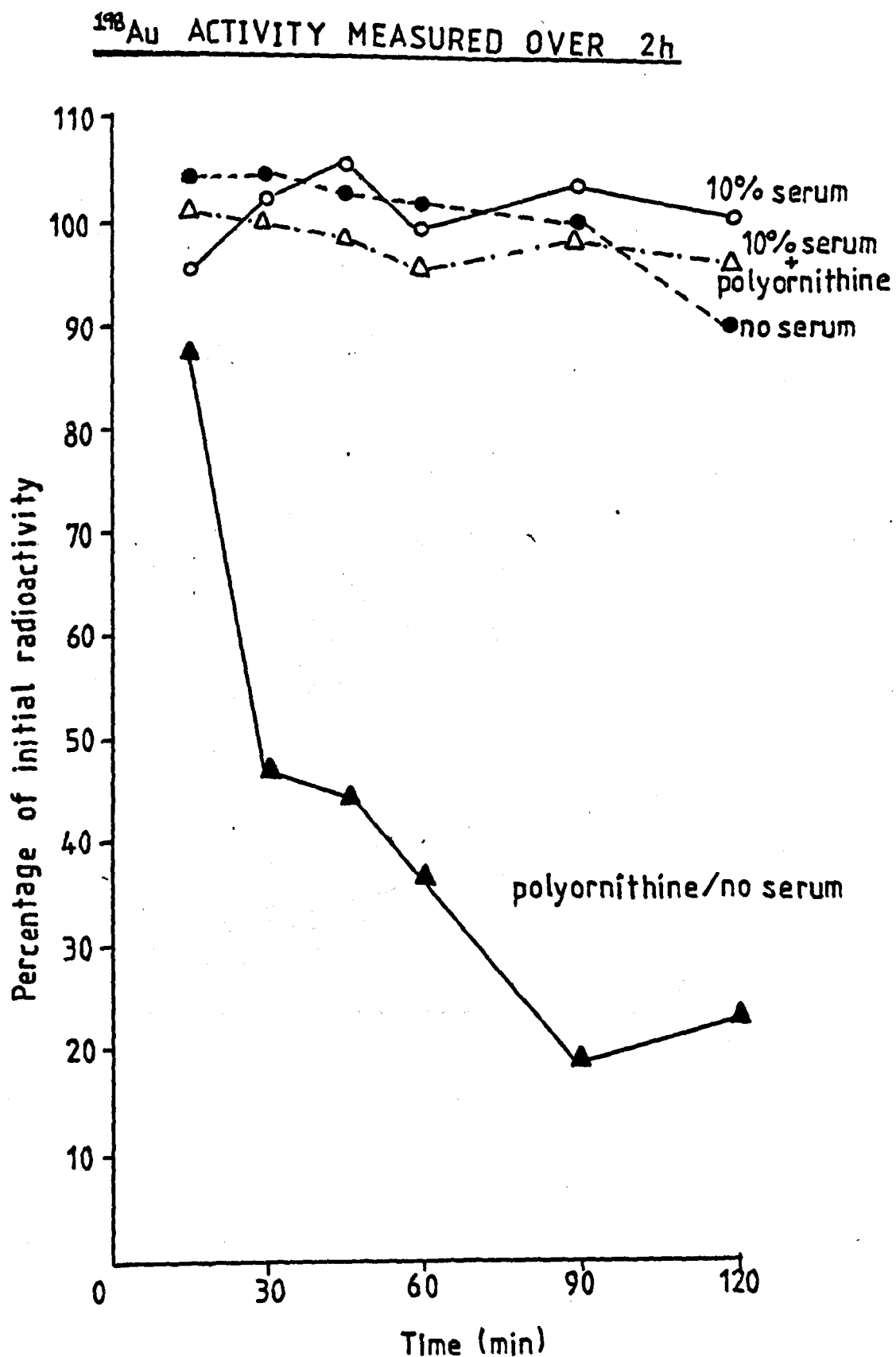
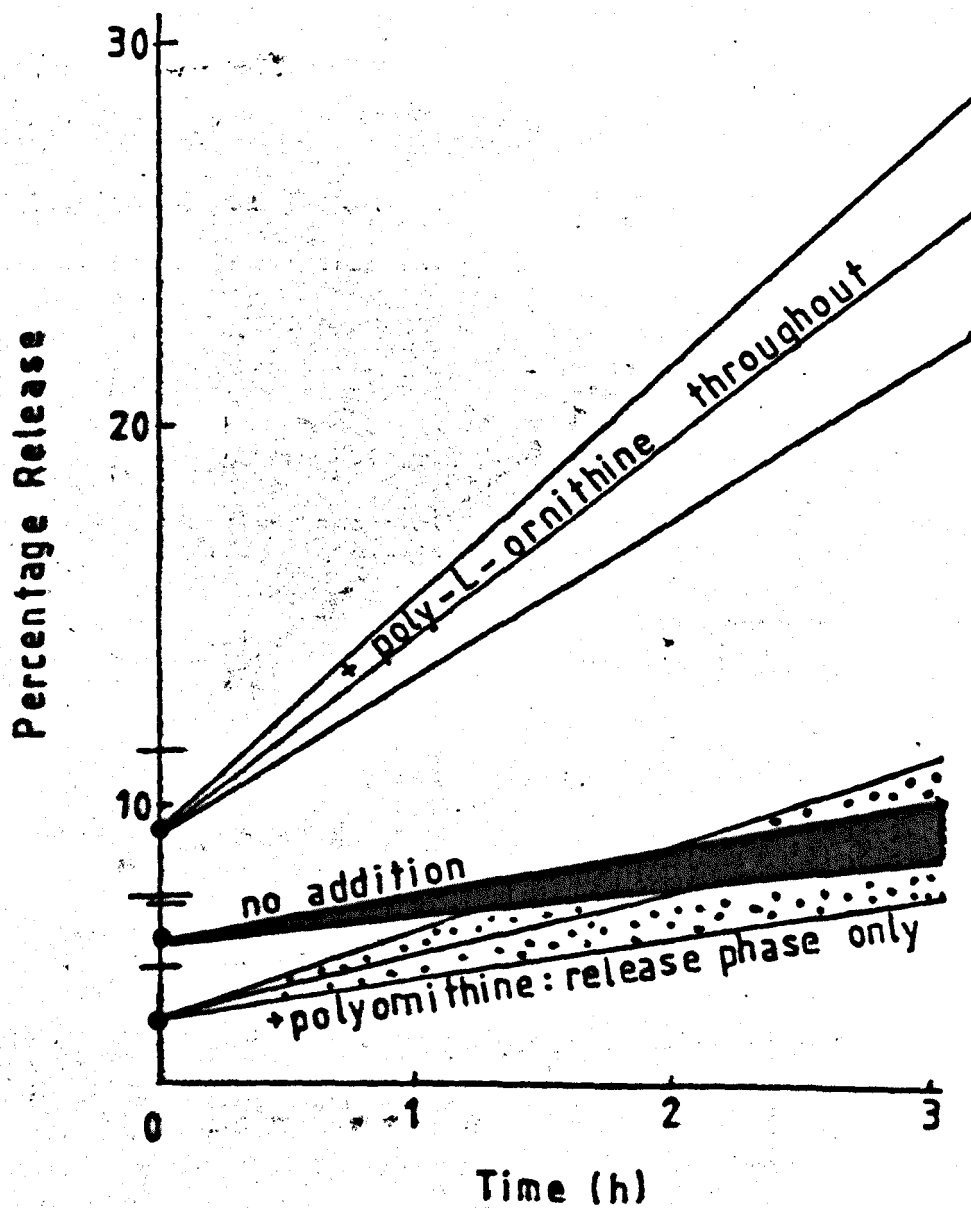


FIG. 6. 8. EXOCYTOSIS OF COLLOIDAL ^{198}Au

(Mean rate of loss \pm S. E.)



6.2.6. Effect of Cationic Polymers on Colloidal ^{198}Au particle size

Samples (1 ml) of medium 199 containing 10% calf serum and colloidal ^{198}Au were subjected to density gradient centrifugation at 5°C .

Fig.6.9. shows the pattern of sedimentation of colloidal ^{198}Au either alone or with additions of poly-L-ornithine ($10\text{ }\mu\text{g/ml}$) or DEAE-dextran ($50\text{ }\mu\text{g/ml}$). Table 6.1. summarizes the radioactivity retrieved from each sucrose layer expressed as a percentage of the activity applied. After 1h centrifugation over 45% of the activity applied in samples containing polymer was below the 2M interface, whereas in control samples the majority of material (88%) had not penetrated this interface. Spinning for a further hour caused a pellet to form in samples containing modifier 90-95% of the activity being in the 2M sucrose layer but had little effect on control samples, in which only a minute pellet was visible, accounting for only 30% of the total activity.

Centrifugation at ambient temperature (closer to the normal culture temperature) produced a similar pattern of sedimentation, but the rate of sedimentation was greater, presumably owing to the lowering of viscosity in the sucrose gradient.

Table 6.2. shows the percentage recoveries of radioactivity in this series of experiments. The recoveries were all relatively high so the results were not biased by loss of activity. The samples producing a pellet showed the lowest recoveries, but this is to be expected as there is always some activity that remains at the bottom of the tube when the pellet is dislodged. To minimize the loss, the last ml of sucrose solution was rinsed around the tube several times until all visible signs of the pellet has disappeared.

FIG. 6.9. DISTRIBUTION OF COLLOIDAL ^{198}Au RADIOACTIVITY FOLLOWING DENSITY GRADIENT CENTRIFUGATION

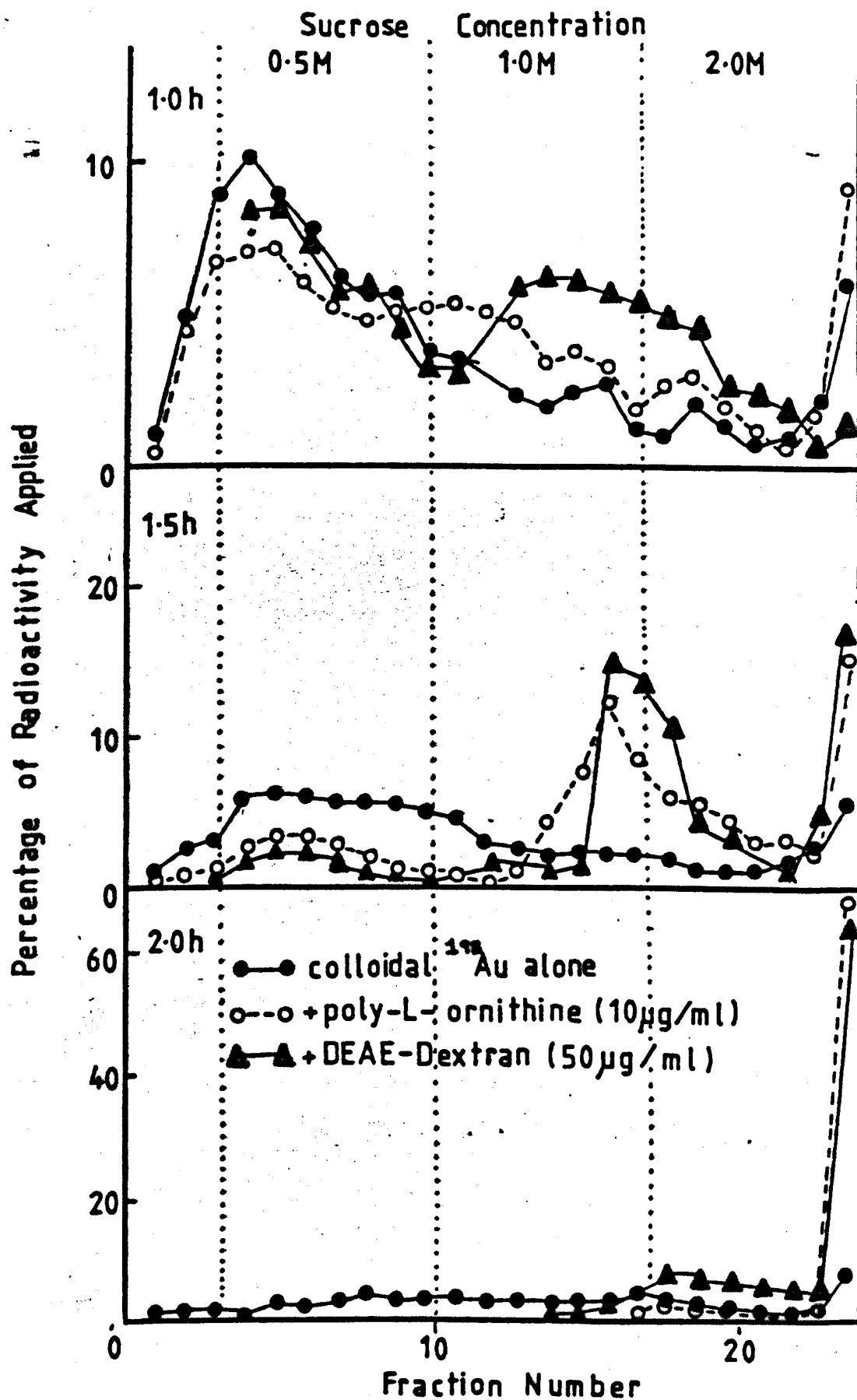


TABLE 6.1. RECOVERY OF COLLOIDAL ^{198}Au RADIOACTIVITY FROM EACH SUCROSE LAYER FOLLOWING CENTRIFUGATION.

| Centrifugation Conditions | Percentage Recovery | | | | |
|--------------------------------------|---------------------|--------------|--------------|--------------|--------------|
| | Sucrose Layer | | | | |
| | 0 | 0.5M | 1.0M | 2.0M | PELLET |
| <u>5°C - 1h</u> | | | | | |
| Colloidal ^{198}Au alone | 15.41 | <u>48.41</u> | 20.24 | 15.96 | - |
| + | | | | | |
| poly-L-ornithine | 11.79 | <u>40.05</u> | 27.23 | 20.92 | - |
| + | | | | | |
| DEAE-dextran | 8.32 | <u>39.38</u> | 38.77 | 13.53 | - |
| <u>5°C-1.5h</u> | | | | | |
| Colloidal ^{198}Au alone | 8.37 | <u>42.96</u> | 27.81 | 20.86 | - |
| + | | | | | |
| poly-L-ornithine | 2.57 | 19.03 | 36.51 | <u>41.85</u> | - |
| + | | | | | |
| DEAE-Dextran | 1.39 | 15.16 | 35.20 | <u>46.23</u> | - |
| <u>5°C - 2h</u> | | | | | |
| Colloidal ^{198}Au alone | 4.77 | 28.72 | <u>35.34</u> | 21.43 | 9.74 |
| + | | | | | |
| poly-L-ornithine | 0.68 | 2.55 | 2.65 | 20.07 | <u>74.07</u> |
| + | | | | | |
| DEAE-dextran | 1.26 | 2.84 | 6.90 | 24.81 | <u>64.17</u> |

The values which are underlined indicate the layer containing most radioactivity for each sample at each time.

TABLE 6.2. TOTAL RECOVERY OF COLLOIDAL ^{198}Au RADIOACTIVITY FROM SUCROSE DENSITY GRADIENTS

| Centrifugation Temperature | Time (h) | Percentage Recovery | | |
|-------------------------------|----------|--------------------------------------|---|---|
| | | Colloidal ^{198}Au Alone | Colloidal ^{198}Au + Poly-L-ornithine | Colloidal ^{198}Au + DEAE Dextran |
| 5°C | 1.5 | 108 | 89 | 138 |
| 5°C | 1.0 | 106 | 99 | 83 |
| 5°C | 2.0 | 92 | 66 | 98 |
| Room Temp. | 1.0 | 100* | 90 | 89 |
| Room Temp. | 1.0 | 100* | 98 100 | - |
| Room Temp. | 0.7 | 100* | 99 96 | - |
| Room Temp. | 0.7 | 100* | 103 83 | - |

* In these experiments the recoveries from gradients loaded with colloidal ^{198}Au alone were substantially greater than 100%. This was due to an underestimation of the radioactivity in the 1ml samples applied initially, as the activity they contained was above the acceptable counting capacity of the gamma counter employed. Thus the recoveries from gradients loaded with samples containing modifier are expressed as a percentage of those loaded with colloidal ^{198}Au alone.

6.3. DISCUSSION

Earlier it has been shown that charged polymers, particularly polycations, modify the rate of uptake of ^{125}I -dBSA and colloidal ^{198}Au by the rat yolk sac (Chapter 5). The inability of the polymers used to modify pinocytosis of ^{125}I -PVP was considered good evidence that the mechanism of pinocytic modification was other than a simple change in the rate of pinosome formation. However, the possibility remains that polycations, when present in the company of ^{125}I -dBSA or colloidal ^{198}Au , might change the rate of vesicle formation. Here (Fig. 6.2.) it is shown that poly-L-ornithine in combination with colloidal ^{198}Au was still unable to alter the Endocytic Index of ^{125}I -PVP, and this confirms the original conclusion that an alteration in the rate of vesicle formation is not an integral part of the mechanism of polycation modification.

Dual-substrate experiments uniquely permit the exact quantitation of the uptake of both substrates into the same tissue at each time interval throughout the culture period. The effect of one substrate on the uptake of another is often studied by adding a cold analogue of one substrate, but this approach is less direct since one is never sure if the analogue will behave in precisely the same way as the radiolabelled form. A cold analogue of colloidal ^{198}Au has been used to show that simultaneous uptake of colloidal ^{198}Au does not effect the rate of pinocytosis of ^{125}I -PVP without addition of modifiers (Roberts et al., 1977), and it is interesting that the dual-substrate experiments reproduce the results obtained. ^{125}I -PVP and colloidal ^{198}Au were taken up simultaneously by the yolk sac with Endocytic Indices of 2.32 and 2.95 $\mu\text{l}/\text{mg}$ protein/h respectively, and these

values are similar to the mean control values for these substrates when measured independently (See Chapter 3).

Various authors have described differences in the stimulatory capacity of the two optical isomers of cationic polyamino acids. Both Ryser & Hancock (1965) and Deierkauf *et al.*, (1977) found poly-D-lysine to be more effective than poly-L-lysine in stimulating endocytosis. However, in contrast Duncan *et al.*, (1979) showed that only poly-L-lysine was able to stimulate uptake by the rat peritoneal macrophage. In the rat yolk sac there was no difference in the stimulatory capacity of the -D- and -L- forms of polylysine. It is perhaps surprising that poly-D-lysine should be the more effective polymer in any system, as this isomer is not degradable and if it is entering the cell rapidly it would soon accumulate in large quantities.

Having established that poly-L-ornithine does not stimulate pinosome formation, even in the presence of colloidal ^{198}Au or the absence of calf serum (Fig 6.6.), further experiments were carried out to investigate the mechanism of the enhancing effect. The addition of metabolic inhibitors to the culture system or a reduction in temperature can completely inhibit the progressive uptake of colloidal ^{198}Au (Chapter 4), although under these conditions there is still some radioactivity associated with the tissue which probably represents bound material. Experiments were carried out using colloidal ^{198}Au as a pinocytic marker in the presence of poly-L-ornithine or DEAE-dextran and in addition the culture included a metabolic inhibitor or were performed at low temperature. In this way the actual quantity of colloidal gold entering the tissue under stimulated conditions was estimated.

Addition of 2,4,-dinitrophenol (50 $\mu\text{g}/\text{ml}$) to cultures with

colloidal ^{198}Au as a marker which contained either poly-L-ornithine or DEAE-dextran did not change the stimulatory capacity of these substances (Figs. 6.3. and 6.4.). As this concentration of inhibitor can completely stop uptake under control conditions, it must be concluded that the enhancement achieved by polycation was entirely due to increased adsorption of substrate onto the outside of the visceral yolk sac with no true pinocytic incorporation.

If the increase in binding of colloidal gold by the visceral yolk sac facilitated by polycations were accompanied by the normal rate of membrane invagination, one would expect to see a component of the uptake process which was sensitive to metabolic inhibition. (Unless the resultant polycation/colloidal ^{198}Au complex was internalized in a different type of vesicle that was not dependent upon a supply of metabolic energy). As this was not the case it must be postulated that either:-

i) Binding of a polycation-colloidal ^{198}Au complex to the plasma membrane prevents membrane internalization.

or

ii) The complex itself is physically too large to enter the pinosomes formed by the yolk sac epithelium.

As ^{125}I -PVP could enter the yolk sac at the normal rate during the stimulation of colloidal ^{198}Au tissue association, this would suggest that binding of the polycation complex to the plasma membrane does not simply prevent all membrane invagination. It does not rule out the possibility that colloidal ^{198}Au enters the cell via a different route and that it is only this pathway that becomes inaccessible during stimulation. (Perhaps colloidal ^{198}Au is too large to enter the vesicles that capture ^{125}I -PVP, but this would not explain why

^{125}I -PVP was excluded from the larger colloidal ^{198}Au vesicles).

Any theory based on the inhibition of membrane internalization seems to present too many problems because of the observation relating to maintained ^{125}I -PVP uptake at all times.

On examination of the colloidal ^{198}Au size in the culture medium using density gradient centrifugation, it was found that poly-L-ornithine and DEAE-dextran caused aggregation. These results contrast with those of Ryser & Hancock (1965) who were unable to find any evidence for such aggregation in their system using a gel filtration method. The discontinuous nature of the sucrose gradient made it difficult to calculate the size of the resultant aggregated particles. Further investigation using a continuous sucrose gradient would refine the data shown here and give the necessary information for determination of aggregate sizes.

'Exocytosis' of colloidal ^{198}Au from stimulated yolk sacs was found to be greater than release from control tissue (Fig. 6.8.). This could possibly indicate that more colloidal ^{198}Au was bound to the plasma membrane of the stimulated tissue, the strength of binding being sufficiently strong to withstand the 6min washing procedure - at least to some extent - but not so permanent as to prevent desorption during the 3h reincubation period. Alternatively, it could suggest that addition of poly-L-ornithine can increase the rate of exocytotic release of colloidal ^{198}Au from the yolk sac epithelium. The latter would seem unlikely as the metabolic inhibitor experiments have already indicated that there is probably little or no colloidal ^{198}Au internalization under stimulated conditions. However, to test out this possibility, yolk sacs were preincubated with colloidal ^{198}Au

and then 10 µg/ml poly-L-ornithine was added during the release phase only. The rate of release of ^{198}Au from these yolk sacs was 2.5%/h, which was not significantly different from the average rate of release from controls (1.5%/h). These results may support the suggestion that poly-L-ornithine simply binds colloidal ^{198}Au to the yolk sac membrane, but it should be pointed out that there are several possible sources of radiolabel released during the "exocytosis" phase of these experiments, and the results are by no means clear cut. Possible sources of released material usually attributed to exocytic release were adequately summarized by Leake and Bowyer (1977):-

1. Leakage from extracellular spaces inaccessible to the washing procedure.
2. Desorption from the dish surface.
3. Cell lysis.
4. Desorption from the cell surface.
5. Exocytosis of previously pinocytosed material.

The mechanism of polymeric stimulation becomes more apparent when all the experimental data are inter-related. Firstly, it should be made clear that poly-L-ornithine and DEAE-dextran do not stimulate the pinocytic uptake of colloidal ^{198}Au by the rat visceral yolk sac, even though there is a marked increase in the tissue association of this substrate when these substances are added to the culture medium. It would seem that in the culture medium there is an interaction between cationic polymers and colloidal ^{198}Au resulting in the formation of an aggregate that has a greater affinity for the plasma membrane than colloidal ^{198}Au alone. Colloidal ^{198}Au is stabilized with a gelatin coat, which has a net negative charge (Horisberger et al.,

1975), so it is not surprising that the negatively charged particles interact with the positively charged residues of the polymers, cross linkages causing aggregation in the culture medium.

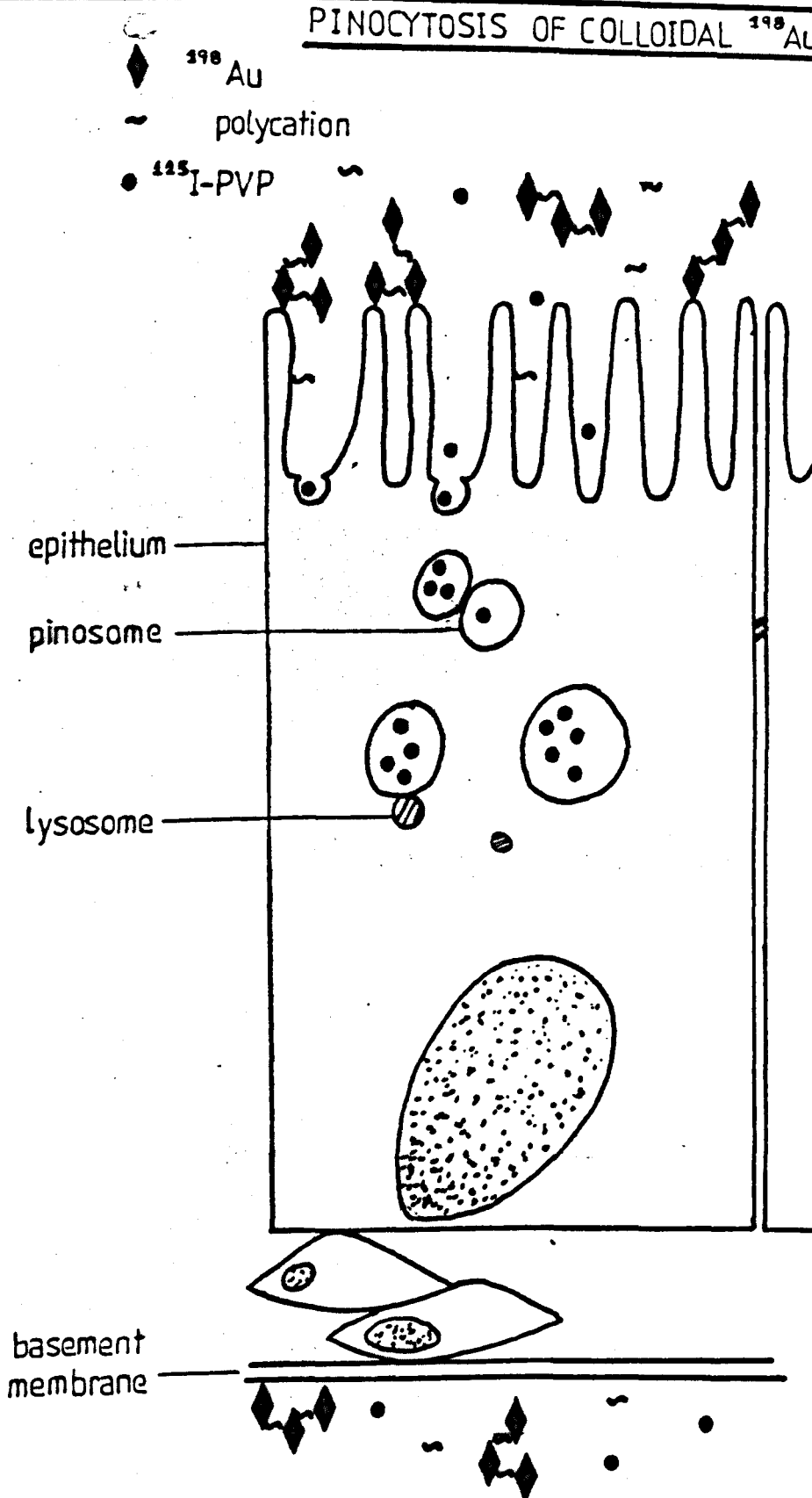
The curves describing uptake against time in Chapter 5 (Figs. 5.4. and 5.5), must now be reinterpreted as binding curves for colloidal ^{198}Au attaching to the yolk sac membrane. Progressive binding occurs over several hours, with saturation after approximately 8h suggesting that the membrane has great capacity for binding the polymer-substrate complex. At 3°C stimulated tissue association was linear with time over the 6.5h culture period for both DEAE-dextran and poly-L-ornithine (Figs. 6.3. and 6.4), but somewhat less than tissue association observed at 37°C . The linearity at low temperature could represent the initial stage in the binding time-course for poly-L-ornithine stimulation, as the overall process would be slowed down by the decrease in temperature. Binding of the DEAE-dextran colloidal ^{198}Au complex was linear with time over the culture period at 37°C , so saturation of the membrane does not occur so quickly. It would be interesting to extend the time course of these experiments and try to define the saturation curve for this aggregate.

As binding of the colloidal ^{198}Au -polymer complex continues for such a long time, it is possible that a multilamellar coat of substrate and modifier could form. The inability of the yolk sac to pinocytose this material could reflect a concentration of the aggregate on non-pinocytic regions of the tissue, such as the basement membrane. This would eliminate all endocytic capture of colloidal ^{198}Au if all of it was in complex form, but it would require a highly selective interaction between the complex and specific membrane components. Alternatively, and more likely, the size of the cation-colloidal ^{198}Au aggregate is too large for it to be captured by the yolk sac epithelium which is known to be composed of cells that are

exclusively pinocytic. Fig. 6.10. is a theoretical model showing the ways that cationic polymers might interact with colloidal ^{198}Au in the yolk sac system. It is noteworthy that the macrophage, which also binds the polymer-colloidal gold aggregate, can internalize this complex (Pratten et al., 1978; Duncan et al., 1979) and this is probably due to the phagocytic capacity of these cells, although the aggregate was shown to be insufficiently large to warrant the induction of truly phagocytic mechanism. In this way polymers increase the quantity of colloidal gold internalized by the macrophage and this directly contrasts with the inhibition of uptake observed in the yolk sac.

The possibility that molecules may specifically alter the rate of uptake of certain substances into defined target tissue may be of great importance in many aspects of cell physiology and medical therapy. Recently it has been shown that the covalent attachment of poly-L-lysine to serum albumin or horseradish peroxidase (Shen & Ryser, 1978) greatly enhances their rate of pinocytosis and extension of this principle led to the formation of a poly-L-lysine-methotrexate complex which was able to penetrate a normally drug resistant cell line (Ryser & Shen, 1978). Polycations have also been shown to enhance the uptake and desulphation of heparin by cultured macrophages (Fabian et al., 1978), a process which is essential in vivo to prevent excessive bleeding following heparin administration. The role of cationic substances in the natural defence mechanism was investigated by Pruzanski and Saito (1978), and they found that cationic subfractions from lysosomes or rabbit polymorphonuclear leucocytes had antibacterial activity. Enhanced bacterial phagocytosis by the polymorphonuclear leucocytes was suggested as a mechanism of action of the cations in

FIG. 6.10. A MODEL TO SHOW THE EFFECT OF POLYCATIONS ON PINOCYTOSIS OF COLLOIDAL ^{198}Au & ^{125}I -PVP



this system.

Here the mechanism of polycation enhancement of pinocytosis of colloidal ^{198}Au in the rat visceral yolk sac was investigated.

It is concluded that polycations do not stimulate pinocytosis as such; in fact, they probably severely hinder the process. Interaction between the modifiers and colloidal ^{198}Au form complexes that bind avidly to the membrane but are not subsequently interiorized.

CHAPTER 7.

ELECTRON MICROSCOPY OF RAT VISCERAL YOLK
SACS FOLLOWING EXPOSURE TO POLYCATIONS.

7.1. INTRODUCTION

In the previous Chapter biochemical data were presented which strongly suggest that the enhanced yolk sac-association of colloidal ^{198}Au observed in the presence of polycations is entirely due to binding of a polycation-colloidal ^{198}Au complex to the outside of the tissue with little or no subsequent internalization. A model was proposed which describes the results obtained (see Fig.69.) Colloidal gold is an electron-dense marker which is frequently used in electron microscopy (Horisberger & Vönlanten, 1979) and it was an obvious step to proceed from the theoretical model based on biochemical information to a morphological approach and hence examine the tissue following polycation stimulation. In this way it was hoped to confirm visually the predictions that were made biochemically.

Electron micrographs presented here represent a preliminary examination of visceral yolk sacs following the polycation-enhanced tissue-association of colloidal gold. The rate of release of colloidal ^{198}Au from the yolk sacs during preparation for electron microscopy was also investigated in order to estimate the quantity of radiotracer that would still be associated with the yolk sac when eventually examined.

7.2. RESULTS

7.2.1. Electron Microscopy of the Visceral Yolk Sac.

A small drop of distilled water containing colloidal gold (100 $\mu\text{g}/\text{ml}$) was allowed to settle on a grid and then examined using the electron microscope. A typical micrograph is shown in Fig. 7.1. and it can be seen that there is quite a range in colloidal gold particle size.

Fig. 7.2. shows the typical appearance of the apical region of a columnar epithelial cell from a yolk sac that had been incubated in the presence of colloidal gold (500 $\mu\text{g}/\text{ml}$) in medium 199 supplemented with 10% calf serum for 3h. Fig. 7.3. shows a similar region derived from another yolk sac which has been incubated for the same length of time under the same conditions, but in the absence of colloidal gold. Cells which were exposed to colloidal gold show a fairly even scatter of electron-dense particles which resemble the colloidal gold particles shown in Fig. 7.1., but are somewhat less distinct in form.

Epithelial cells derived from yolk sacs incubated as above with colloidal gold (500 $\mu\text{g}/\text{ml}$) and poly-L-ornithine (10 $\mu\text{g}/\text{ml}$) are shown at various magnifications in Figs. 7.4. - 7.6. At low magnification (7.4) there is no evidence of a dense layer of colloidal gold in association with the exterior of these cells. Higher magnification reveals an even scatter of dense particles throughout the apical region of the cell, a very similar appearance to that observed in cells incubated with colloidal gold but not poly-L-ornithine.

The appearance of cells incubated in the presence of colloidal gold (500 $\mu\text{g}/\text{ml}$) and DEAE-dextran (50 $\mu\text{g}/\text{ml}$) was typically similar to those shown that were incubated with poly-L-ornithine. Fig. 7.7.

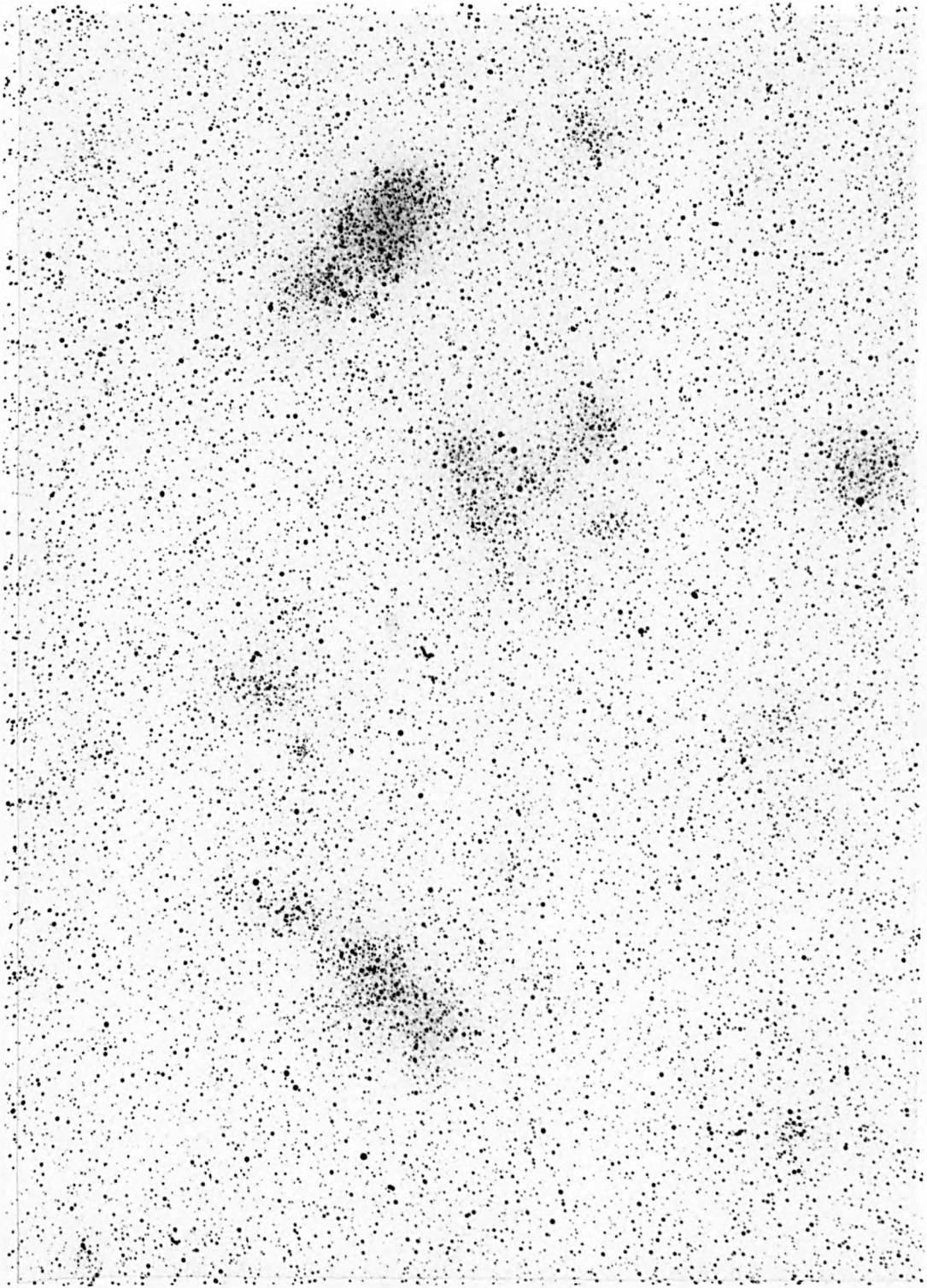


FIG. 7.1. COLLOIDAL GOLD (100 μ g/ml)

magnification x 72,400



FIG.7.2. THE APICAL REGION OF A COLUMNAR EPITHELIAL
CELL INCUBATED IN THE PRESENCE OF COLLOIDAL
GOLD (500 μ g/ml) magnification x 72,400

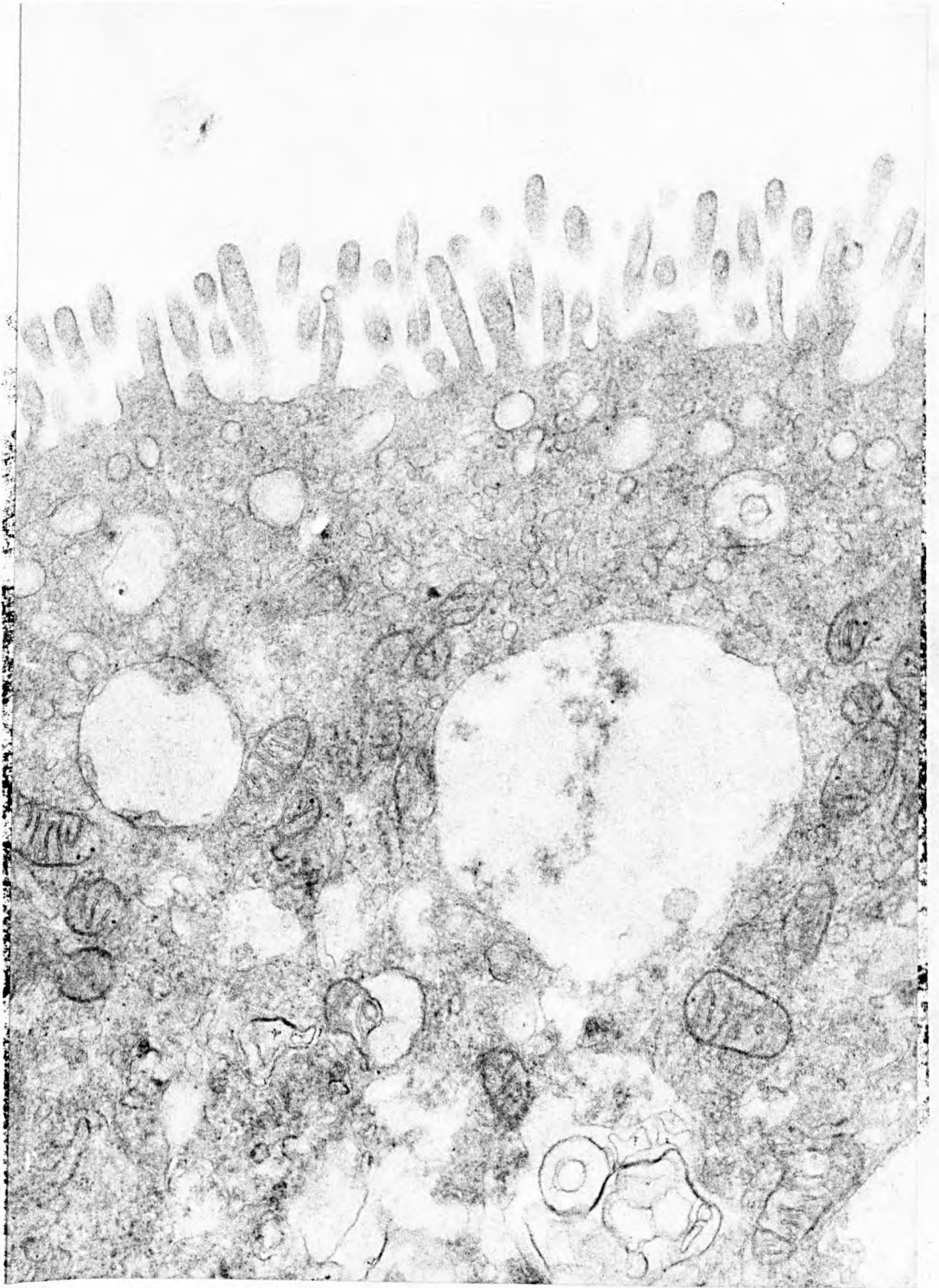


FIG. 7.3. THE APICAL REGION OF A COLUMNAR
EPITHELIAL CELL INCUBATED IN THE
ABSENCE OF COLLOIDAL GOLD magnification
x 25,000

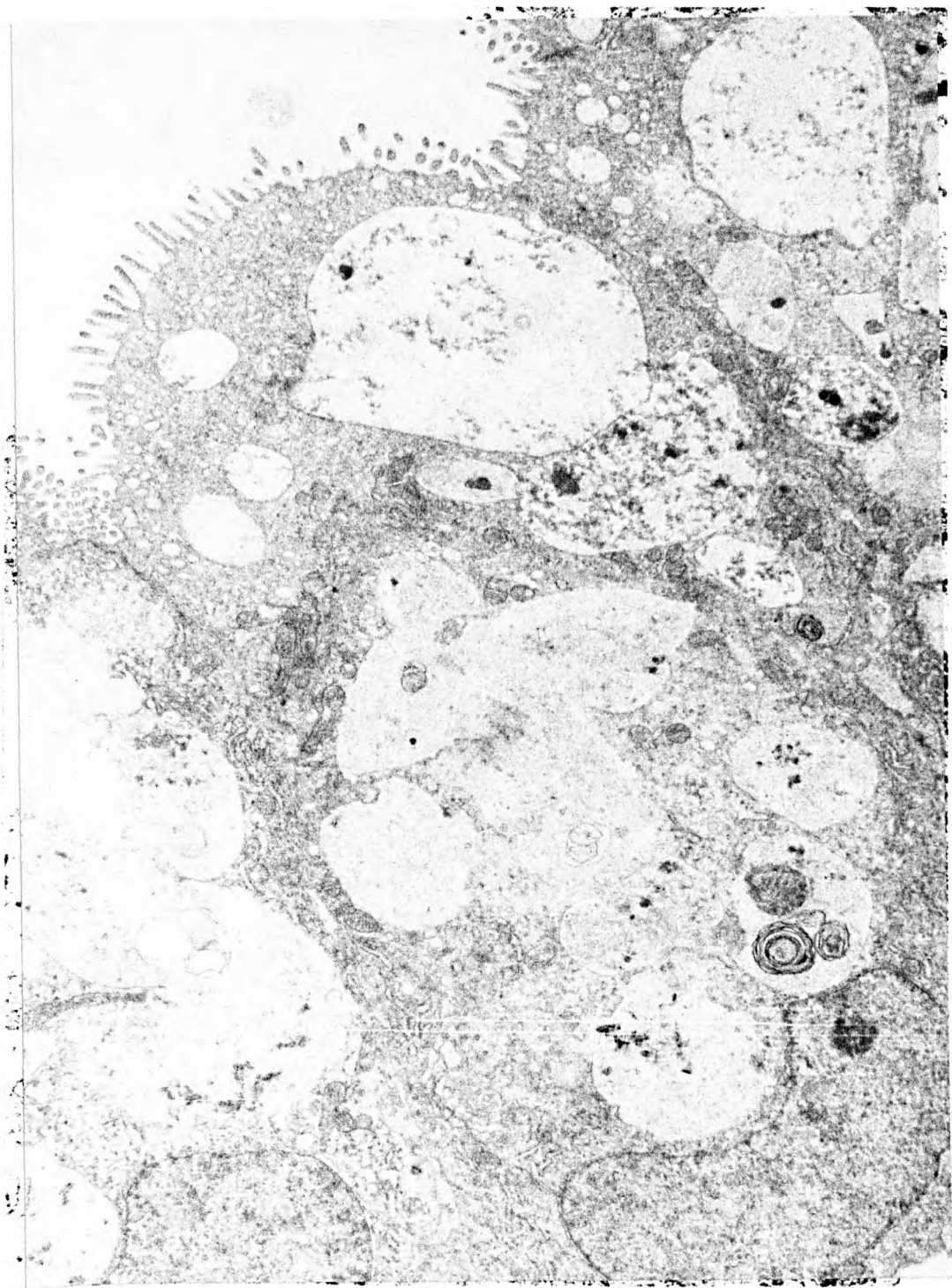


FIG. 7.4. COLUMNAR EPITHELIAL CELLS INCUBATED
WITH COLLOIDAL GOLD (500 μ g/ml) AND
POLY-L-ORNITHINE (10 μ g/ml) magnification
x 6,500

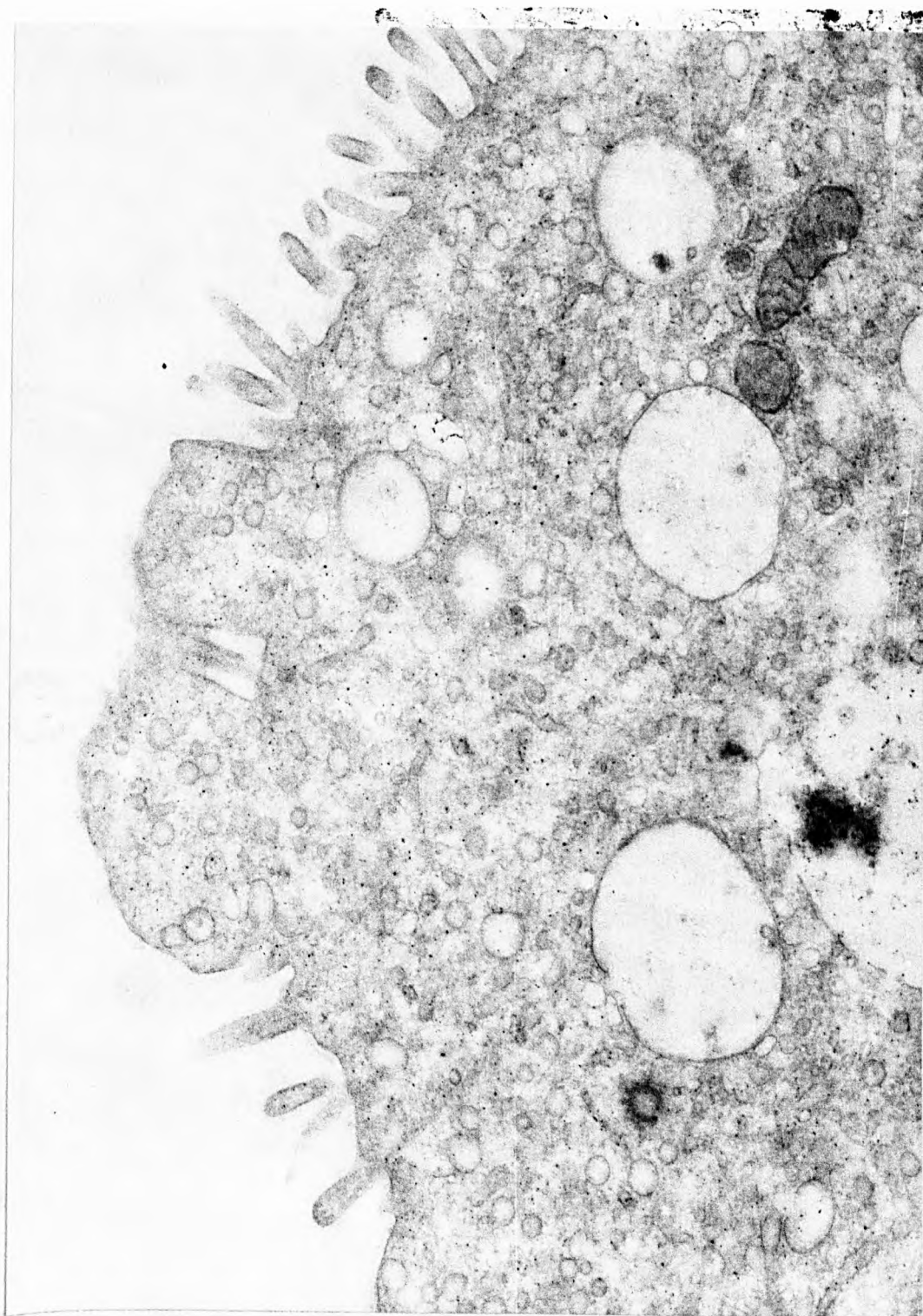


FIG. 7.5. APICAL REGION OF AN EPITHELIAL CELL
INCUBATED WITH COLLOIDAL GOLD (500 μ g/ml)
AND POLY-L-ORNITHINE (10 μ g/ml)
magnification x 25,000

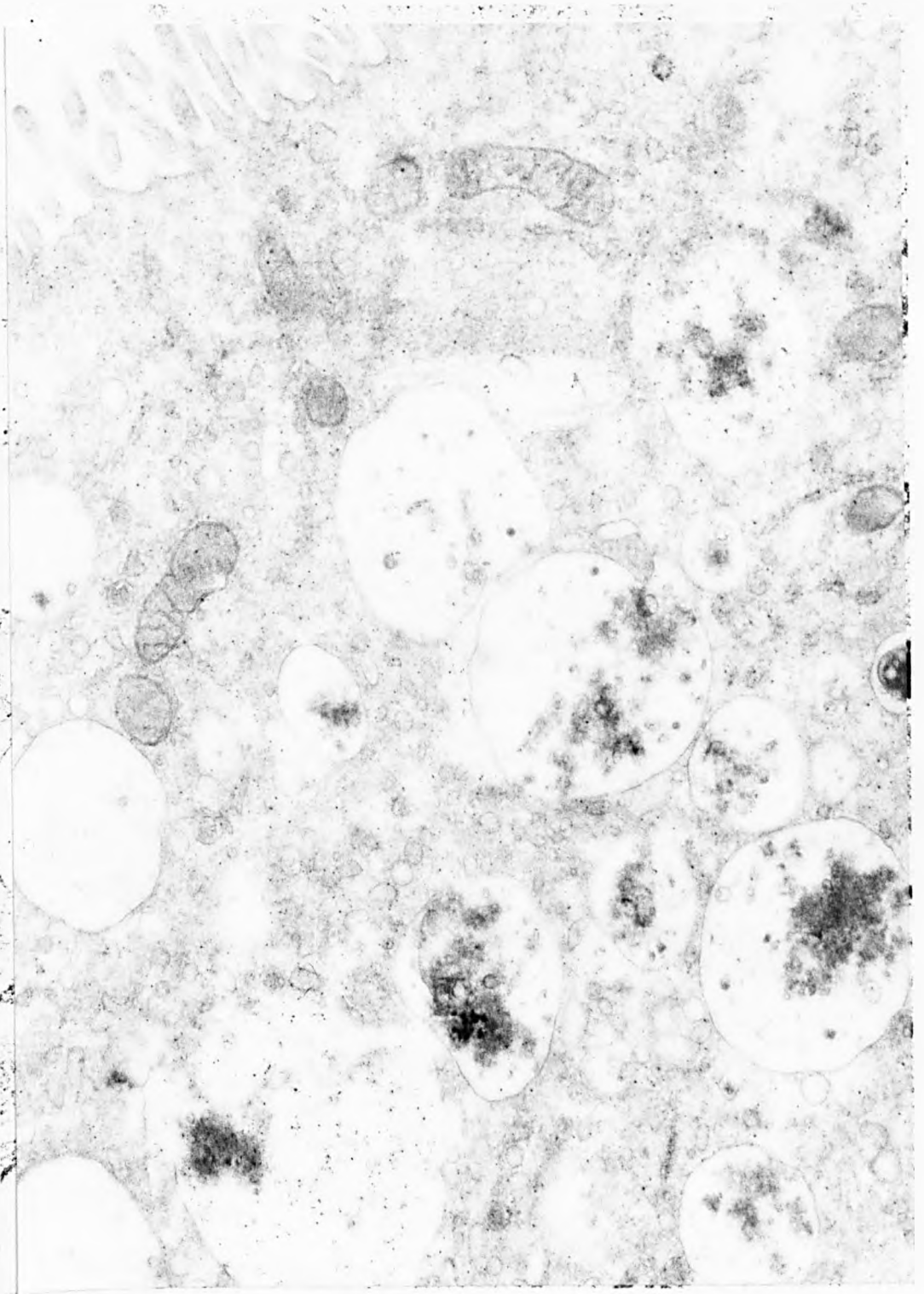


FIG. 7.6. APICAL REGION OF AN EPITHELIAL CELL
INCUBATED WITH COLLOIDAL GOLD (500 μ g/ml)
AND POLY-L-ORNITHINE (10 μ g/ml)
magnification x 25,000

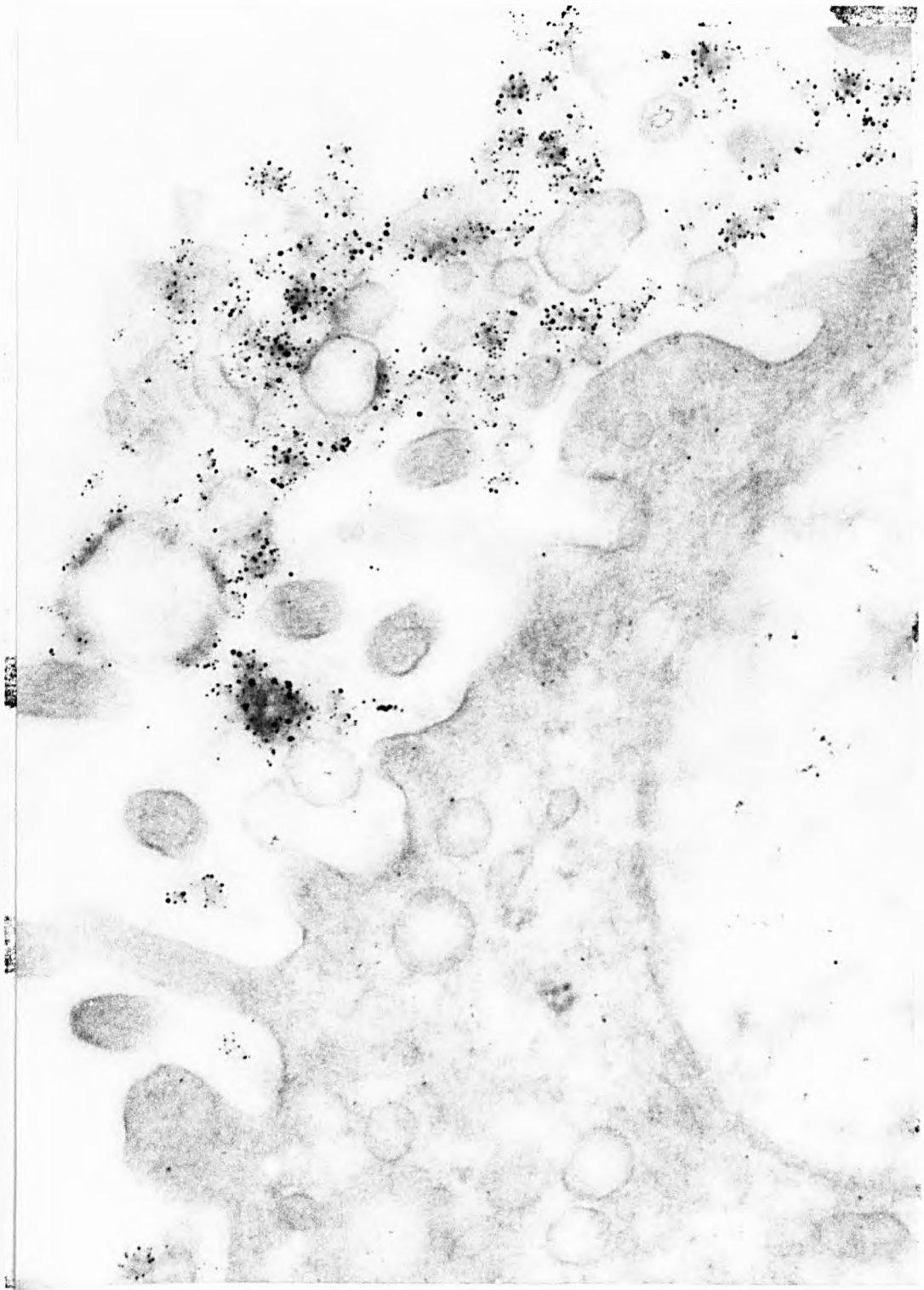


FIG. 7.7. AN ATYPICAL APICAL REGION OF AN
EPITHELIAL CELL INCUBATED WITH
COLLOIDAL GOLD (500 μ g/ml) AND DEAE-
DEXTRAN (50 μ g/ml) magnification x72,400

shows an atypical apical region in addition there appears to be a considerable quantity of colloidal gold associated with the apical microvilli, as well as the scattering of particles visible within the cell.

7.2.2. Release of Colloidal ^{198}Au From Visceral Yolk Sacs During Preparation for Electron Microscopy

Visceral yolk sacs were cultured in medium 199 and 10% calf serum for 3h with colloidal ^{198}Au (20 $\mu\text{g}/\text{ml}$) alone or with colloidal ^{198}Au (20 $\mu\text{g}/\text{ml}$) and either poly-L-ornithine (10 $\mu\text{g}/\text{ml}$) or DEAE-dextran 50 $\mu\text{g}/\text{ml}$). They were then removed from the culture flasks and placed immediately in glutaraldehyde fixative. Over the following 7h they were treated as if they were to be prepared for electron microscopy and the procedures they underwent are described in Table 7.1. Duplicate 1ml samples of the solutions used at each stage were assayed for radioactivity and the loss of colloidal ^{198}Au at each stage was expressed as a percentage of the total tissue-associated activity present after the 3h incubation. These values are shown in Table 7.2. and an overall summary of the data shown in Table 7.3.

After preparation for electron microscopy the yolk sacs were not readily soluble, so protein determination was difficult. A crude estimate of protein in each yolk sac was obtained by firstly dissolving the tissue in boiling Domestos (5ml), and then following the usual procedure (see Section 2.2.). The mean value was 7.08mg protein per yolk sac and this was used to obtain an estimate of the tissue-association of colloidal ^{198}Au that occurred during the 3h incubation phase (Table 7.4.).

TABLE 7.1. PROCEDURE EMPLOYED FOR QUANTITATION OF COLLOIDAL ^{198}Au
RELEASE DURING PREPARATION FOR ELECTRON MICROSCOPY

| Stage | Procedure | Length of Treatment | Solution Volume |
|-------|-------------------------|---------------------|-----------------|
| 1 | Glutaraldehyde Fixation | 3h | 3ml |
| 2 | Buffer Rinse | 20min | " |
| 3 | Buffer Rinse | 30min | " |
| 4 | Osmium Fixation | 45min | " |
| | Dehydration: | | |
| 5 | 30% Acetone | 15min | " |
| 6 | 60% Acetone | 15min | " |
| 7 | 90% Acetone | 15min | " |
| 8 | 100% Acetone | 45min | " |

Details of the solutions used in each step are shown in Section 2.11.

TABLE 7.2. RELEASE OF COLLOIDAL ^{198}Au FROM YOLK SACS AT VARIOUS STAGES OF PREPARATION FOR ELECTRON MICROSCOPY.

| Treatment | Experiment | Stage (See Table 7.1.) | | | | | | | |
|------------------|------------|------------------------|-----|-----|-----|-----|-----|-----|------|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| NONE | A | 56.8 | 2.9 | 0.3 | 0.2 | 0.1 | 0.0 | 0.0 | 0.0 |
| | B | 54.0 | 7.4 | 0.6 | 0.5 | 0.2 | 0.2 | 0.2 | 0.0 |
| POLY-L-ORNITHINE | A | 44.8 | 3.7 | 0.5 | 0.3 | 0.2 | 0.3 | 0.0 | 0.0 |
| | B | 45.1 | 4.2 | 0.7 | 0.7 | 0.5 | 0.1 | 0.2 | - |
| DEAE-DEXTRAN | A | 15.0 | 1.8 | 0.7 | 0.4 | 0.7 | 0.5 | 0.5 | 0.1 |
| | B | 14.6 | 1.5 | 0.4 | 0.6 | 0.2 | 0.3 | 0.2 | 0.2. |

Figures show percentage of initial tissue-associated radioactivity released at each stage of preparation.

TABLE 7.3. SUMMARY OF RELEASE OF RADIOACTIVITY FROM YOLK SACS DURING PREPARATION FOR ELECTRON MICROSCOPY.

| Treatment | Mean Loss at Stage 1 (%) | Mean Total Loss (%) | Mean Rate of Loss Over the Stages of Preparation (%/h) |
|------------------|--------------------------|---------------------|--|
| NONE | 55.4 | 61.7 | 0.94 |
| POLY-L-ORNITHINE | 45.0 | 50.6 | 0.82 |
| DEAE-DEXTRAN | 14.8 | 18.9 | 0.61 |

TABLE 7.4. TISSUE ASSOCIATION OF COLLOIDAL ^{198}Au ACHIEVED DURING THE 3h INCUBATION BY THE YOLK SACS USED FOR THE STUDY OF RELEASE OF COLLOIDAL ^{198}Au DURING PREPARATION FOR ELECTRON MICROSCOPY.

| Treatment | Experiment | Uptake after 3h ($\mu\text{l}/\text{mg}$ protein) |
|------------------|------------|--|
| NONE | A | 32.09 |
| | B | 44.68 |
| POLY-L-ORNITHINE | A | 44.57 |
| | B | 29.46 |
| DEAE-DEXTRAN | A | 196.89 |
| | B | 191.01 |

7.3. DISCUSSION

Colloidal gold of average particle size 10nm (100 $\mu\text{g/ml}$) is readily visible with the electron microscope and appears as small spherical dense particles at high magnification (Fig.7.1.). Following incubation in the presence of colloidal gold the visceral yolk sac epithelial cells contain many small electron dense particles which resemble colloidal gold although they appear to have a slightly different texture, and these particles were not detectable if the tissue was incubated in colloidal gold-free medium (Fig.7.2.). However, it is puzzling that these particles are not restricted to the interior of membrane-bounded profiles and they are also apparent in the cytoplasm and microvilli. There was no obvious association of this material with the external surface of the plasma membrane which might have been expected as it is known that colloidal gold is pinocytosed at least partially by an adsorptive mechanism.

Addition of polycation to the culture medium did not cause the appearance of a colloidal gold "coat" around the surface of the yolk sac cells, an event that was predicted on the basis of the biochemical data reported in Chapter 6. These cells in fact resembled those observed in the absence of modifier, with the colloidal gold-like particles visible intracellularly. In a single instance following DEAE-dextran stimulation there was a substantial quantity of particulate matter seen in association with the microvilli (Fig.7.7.).

Although the electron microscopy of visceral yolk sacs following polycation stimulation did not verify the mechanism of action proposed in Chapter 6, neither do the results obtained discount such a mechanism. This becomes increasingly obvious if one considers the extensive preparative procedures that the experimental material undergoes before it is suitable for visualization. Material that is externally

disposed on any yolk sac surface could easily be dislodged during these events, so a colloidal ^{198}Au experiment was carried out using the radioactive marker to quantitate the amount of tracer remaining with the tissue after preparation for electron microscopy. It was found that approximately 60% of the colloidal ^{198}Au accumulated by yolk sacs cultured with no addition was lost during the preparative stages, 50% of the radioactivity from poly-L-ornithine-stimulated yolk sacs was lost and 20% of the radioactivity from DEAE-dextran stimulated yolk sacs was also lost. Relating back to the quantities of radiolabel that became tissue-associated during incubation under these various conditions (3h incubation) it is possible to estimate the amounts (μg) of colloidal ^{198}Au that are present in the tissue when it is finally examined under the microscope (see Table 7.4.). After incubation in either colloidal gold alone or with addition of poly-L-ornithine (10 $\mu\text{g}/\text{ml}$) approximately 2 μg of marker remained associated with the entire tissue before visualization. Addition of DEAE-dextran (50 $\mu\text{g}/\text{ml}$) elevated the amount of marker that remained tissue-associated to approximately 22 μg . Assuming all the colloidal gold particles have a diameter of 10nm there are approximately 10^{11} particles/ μg , and assuming the visceral yolk sac is spherical with a diameter of 1cm and thickness of 20 μ the number of colloidal gold particles that might be available for visualization in any section can be estimated. Using such crude approximations it appears that something in the order of 20 particles should be present per sq μm when the tissue is incubated in colloidal gold alone or with an addition of poly-L-ornithine. Addition of DEAE-dextran would raise this number by a factor of ten so this might explain why one area of microvillous surface was found which did reveal an aggregation of

colloidal gold. As the visceral yolk sac is known to be thrown into many folds these calculations are likely to be a considerable over-estimation of colloidal gold density one might expect to see on the micrographs.

On reflection the expectation that one might be able to use the electron microscope to examine the yolk sac epithelial cells and visualize the events proposed in Chapter 6 following administration of polycation to the system when using colloidal gold as a pinocytic marker may have been rather naïve. There are obvious technical problems in getting high enough concentrations of colloidal gold to become tissue-associated even when the tissue is exposed to high concentrations of the marker during incubation. Although relatively little material is lost during the lengthy preparation for electron microscopy, up to 50% of the radioactive colloidal gold was released during immersion in the first solution. It is interesting to note that yolk sacs exposed to DEAE-dextran released significantly less material at this stage. In the initial series of experiments (Chapters 5 & 6) colloidal ^{198}Au was used at a concentration of $1\text{ }\mu\text{g/ml}$, whereas the concentration was raised to $20\text{ }\mu\text{g/ml}$ when trying to examine the rate of release from treated yolk sacs. This may explain why the uptake values reported in Table 7.4. are not consistent with those reported in Chapters 5 & 6. Uptake measured in the presence of poly-L-ornithine was not any higher than that of colloidal ^{198}Au measured alone. In contrast stimulated uptake seen in the presence of DEAE-dextran was many times higher than that reported in Chapter 6. These differences may well reflect the importance of the colloidal Au-polycation ratio in the culture medium and also the saturability of the yolk sac itself. Elevation of the substrate concentration not only disturbs this balance in terms of the time length for substrate/yolk sac interaction during

culture but also greatly affects the polycation-colloidal gold ratio.

Even though some of the technical problems encountered are probably insurmountable, this short electron microscopical study does make a worthwhile contribution to this thesis. It should be stressed that whilst the observations made in this Chapter do not reinforce the conclusions drawn in Chapter 6, they certainly do not make them invalid. In fact observations made here are not totally negative as it is now known that visceral yolk sacs incubated for 3h in the presence of poly-L-ornithine ($10\mu\text{g/ml}$) and DEAE-dextran ($50\mu\text{g/ml}$) do not differ in appearance from yolk sacs cultured alone for that length of time, a fact which reinforces the assumption that these modifiers are not cytotoxic at the concentrations employed. The technical difficulties which made the marriage of biochemical data and morphological observation impossible illustrate the impracticality of the visual approach in certain systems. The electron-dense marker seems to be required in such high concentrations during loading of the tissue that it is quite likely that the viability of the cells will be impaired.

CHAPTER 8.

EFFECT OF DIVEMA AND DIVEMA DERIVATIVES ON PINOCYTOSIS.

8.1. INTRODUCTION

Divinyl ether-maleic anhydride (DIVEMA) is a synthetic anionic polyelectrolyte sometimes known as pyran copolymer, (Fig 8.1. and 8.2) which has been shown to display a wide variety of biological activities. These activities have recently been reviewed by Breslow(1976) and are summarized in Table 8.1. The possibility that one of DIVEMA's primary effects was on the immune response was investigated by Munson et al., (1970). They examined the effect of DIVEMA on the reticuloendothelial system and it was concluded that administration of DIVEMA caused an inhibition of phagocytosis 2 days after injection, but a threefold stimulation of phagocytosis by 6 - 8 days. Breslow et al., (1973) examined this effect further and postulated that the biphasic phagocytic response was caused by the differential effects of a low molecular weight polymer (the stimulator) and high molecular weight polymer (the inhibitor) present within the DIVEMA sample injected. They tested this hypothesis by synthesizing various molecular weight ranges with narrow distributions and investigating the effect of these fractions on the clearance of carbon from mice 24h after injection. Molecular weights between 2,500 and 15,000 stimulated uptake maximally 2-3 fold, whereas high molecular weight species inhibited uptake to less than 25% the control level.

DIVEMA has also been used as a carrier for methotrexate, the dihydrofolate reductase inhibitor that is one of the most widely used drugs in the treatment of neoplastic diseases. The DIVEMA methotrexate complex was synthesized with the aim of combining antineoplastic and immune stimulating activity and the polymer complex produced was more potent than methotrexate alone against murine leukemia and solid tumour (Przybylski et al., 1978 a,b). The effects

FIG 8.1. STRUCTURE OF DIVEMA

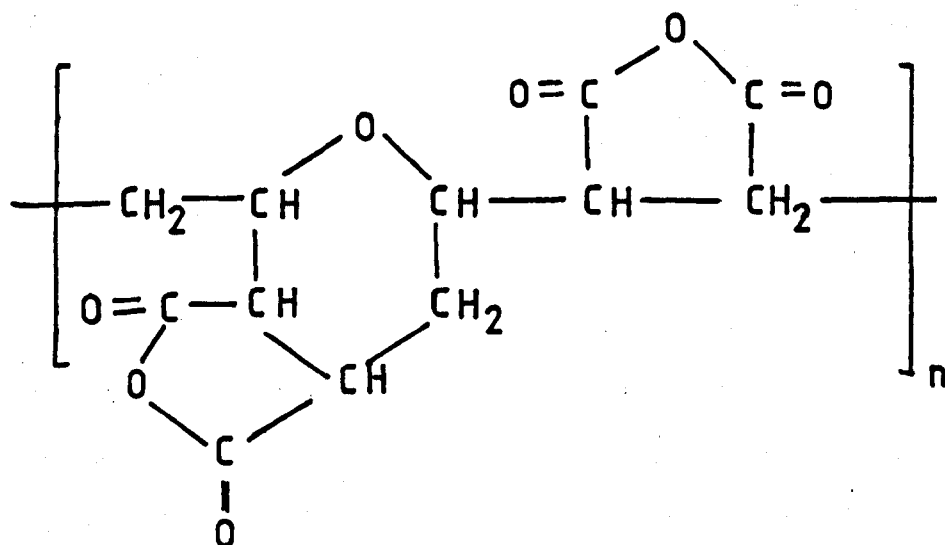


FIG. 8.2.

Hydrolysed DIVEMA :

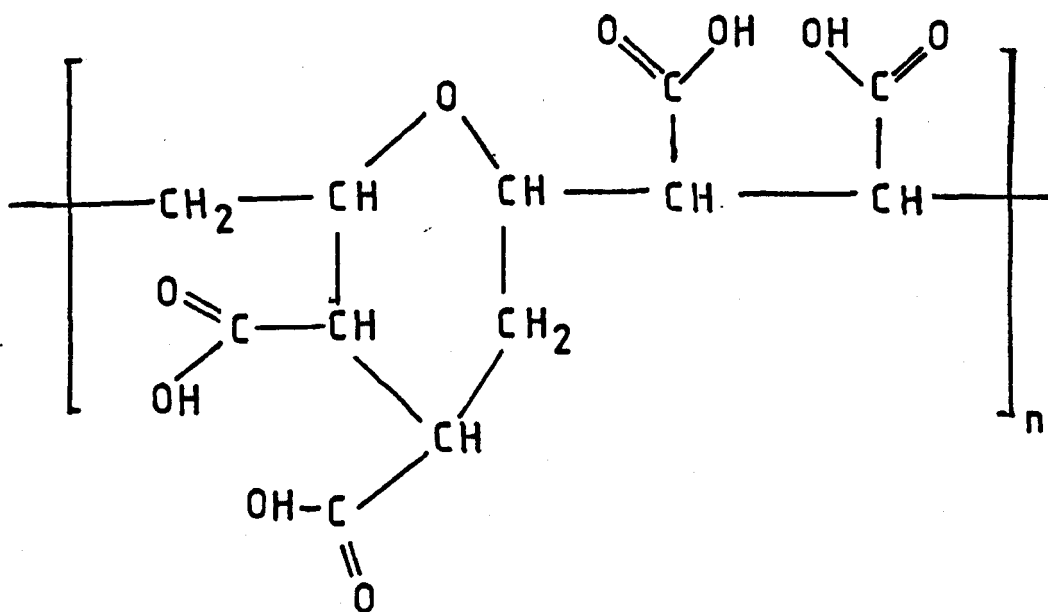


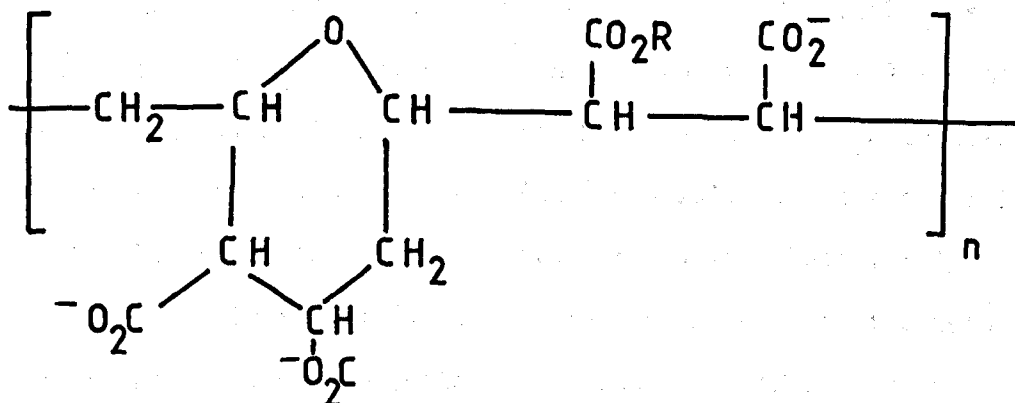
TABLE 8.1. BIOLOGICAL ACTIVITY OF DIVEMA

| | |
|------------------------------------|---------------------------------|
| Antitumour | Morahan <u>et al.</u> , (1974) |
| Interferon inducer | Merigan (1967) |
| Antiviral | Chirigos <u>et al.</u> , (1969) |
| Antibacterial | Regelson & Munson (1970) |
| Antifungal | " " |
| Anticoagulant | Roberts <u>et al.</u> , (1973) |
| Inhibits reverse transcriptase | Papas <u>et al.</u> , (1974) |
| Activates macrophages | Kaplan <u>et al.</u> , 1974) |
| Elimination of polymeric plutonium | Baxter <u>et al.</u> , (1973) |

of DIVEMA on endocytosis are therefore of additional interest as a DIVEMA methotrexate complex is likely to be taken up by endocytosis, whereas methotrexate itself is interiorized by active carrier-mediated transport (Goldman, 1971).

The study described in this Chapter was an investigation of the effect of three different molecular weight DIVEMAs (4,000, 18,000 and 24,000) on the pinocytosis of ^{125}I -PVP and colloidal ^{198}Au by the rat yolk sac. The effect of three DIVEMA derivatives was also investigated; these were, a DIVEMA with a content of 10 - 14% pyrrolidone, a DIVEMA with a content of 10-14% betaine-structure, and also a DIVEMA 10 - 14% of hydrophobic groups. The characteristics and code numbers of the substances used are shown in Table 8.2. All these compounds were kindly donated by Professor H. Ringsdorf, University of Mainz.

TABLE 8.2. CHEMICAL DATA ON DIVEMA SAMPLES USED



| Substance No. | Hercules Inc designation | Mean Molecular weight | Substituent R |
|---------------|--------------------------|-----------------------|---------------|
| S9 | X 199 10-4 | 4,400 | H |
| S10 | X 204 39-43 | 11,000 | H |
| S11 | X 207 06-45 | 24,000 | H |
| S12 | - | - | |
| S13 | - | - | |
| S14 | - | - | |

S9 - S11 were prepared and characterized by D Breslow and co-workers, Hercules Inc., Wilmington DE 19899, U.S.A.

S12 - S14 were prepared by reaction of DIVEMA (Hercules designation XA 146 - 85 - 2, mean molecular weight 16,800 with different alcohols)

8.2. RESULTS

Quantitation of ^{125}I -PVP and colloidal ^{198}Au uptake was carried out as described in Chapter 2. The DIVEMAs were hydrolysed before use by dissolving in 2% sodium bicarbonate and leaving to stand for 30min. (The hydrophobic DIVEMA (Sl3) was initially dissolved in 100 μ l of DMF by gently shaking the container and then left for 15min before addition of bicarbonate). They were then made up to the required volume with medium 199 (with or without 10% calf serum) and kept at 2°C until required. The final concentration of sodium bicarbonate was 0.02%, so the control experiments without DIVEMA contained the same concentration of sodium bicarbonate. DIVEMA was added simultaneously with the substrate and incubations carried out for periods up to 7h.

8.2.1. Uptake of ^{125}I -PVP in the Presence of DIVEMAS and DIVEMA

Derivatives.

Uptake of ^{125}I -PVP was linear with time and Table 8.3. shows that the Endocytic Indices measured in the presence of DIVEMAS are not appreciably different from the values measured in their absence. Figs 8.3. and 8.4. confirms these observations for the unmodified and modified DIVEMAS respectively and demonstrates the linearity of uptake.

Investigation of the effect of DIVEMAS on the uptake of ^{125}I -PVP under serum-free condition raised a certain problem. Cultures containing no DIVEMA showed a considerably lower and inconsistent accumulation of ^{125}I -PVP than that usually observed under these conditions and this was attributed to an inhibitory effect of the 0.02% sodium bicarbonate. Although the DIVEMA cultures initially contained this concentration of bicarbonate, varying proportions would be utilized in the hydrolysis step, so in fact the control undertaken is not altogether suitable. In Table 8.4.

TABLE 8.3. SUMMARY OF EFFECTS OF DIVEMAs ON THE UPTAKE OF ^{125}I -PVP
IN THE PRESENCE OF 10% CALF SERUM

| <u>SUBSTANCE</u> | <u>CONCENTRATION</u> | | |
|------------------|-----------------------|------------------------|------------------------|
| | 10 $\mu\text{g/ml}$ | 50 $\mu\text{g/ml}$ | 100 $\mu\text{g/ml}$ |
| S9D | 1.66 \pm 0.05 (75%) | 2.56 \pm 0.28 (96%) | 2.37 \pm 0.17 (124%) |
| S10D | - | 2.01 \pm 0.13 (101%) | - |
| S11D | 1.79 \pm 0.26 (89%) | 1.69 \pm 0.20 (85%) | 1.93 \pm 0.08 (97%) |
| S12M | 2.4 \pm 0.04 (118%) | 2.00 \pm 0.26 (76%) | 2.08 \pm 0.31 (103%) |
| S13M | 1.80 \pm 0.34 (99%) | 1.87 \pm 0.24 (104%) | X |
| S14M | 1.73 \pm 0.26 (81%) | 2.19 \pm 0.04 (102%) | 1.70 \pm 0.26 (90%) |

X Not soluble.

FIG. 8.3. EFFECT OF DIVEMA ON THE UPTAKE OF 125 I-PVP

- 10 μ g/ml
- 50 μ g/ml
- △---△ 100 μ g/ml

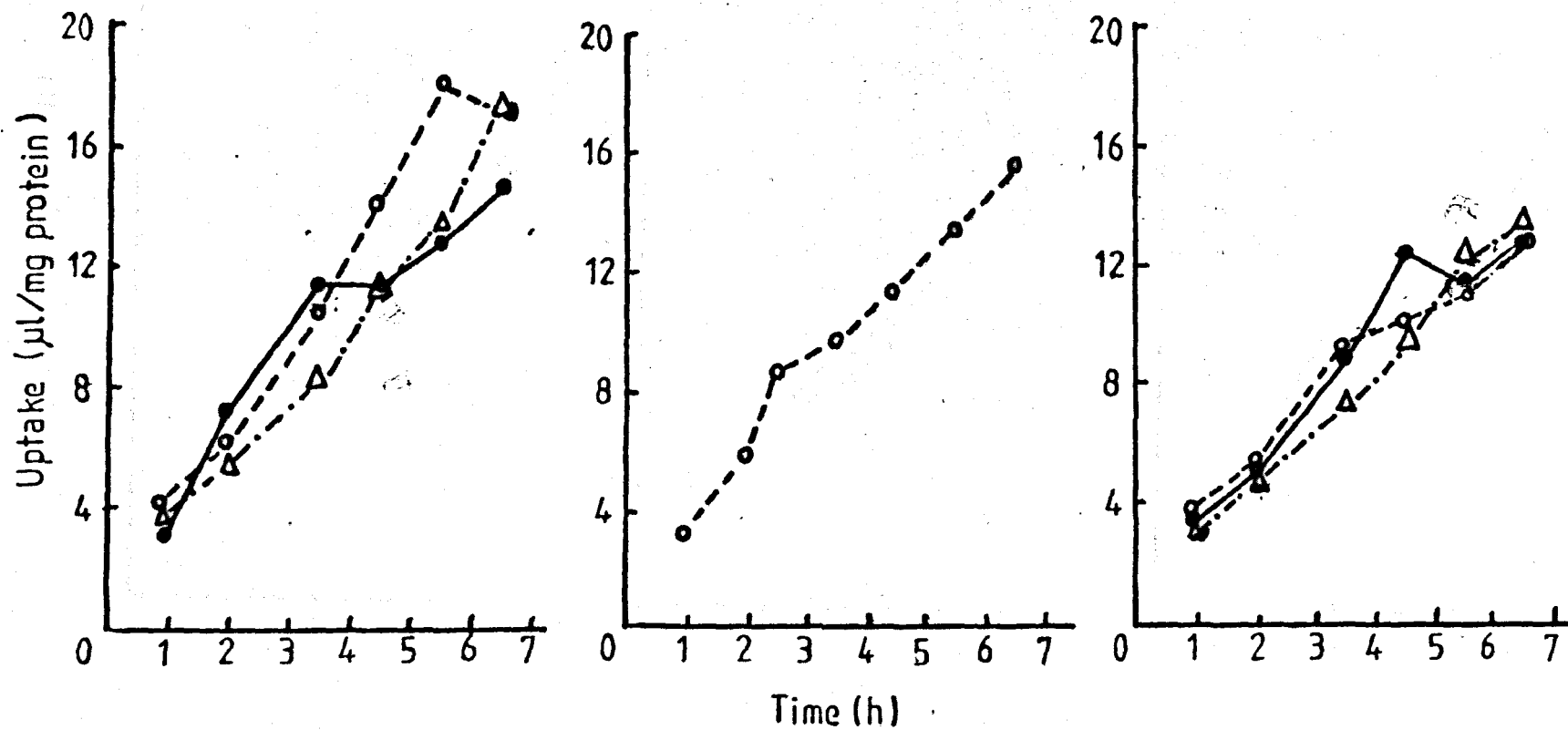
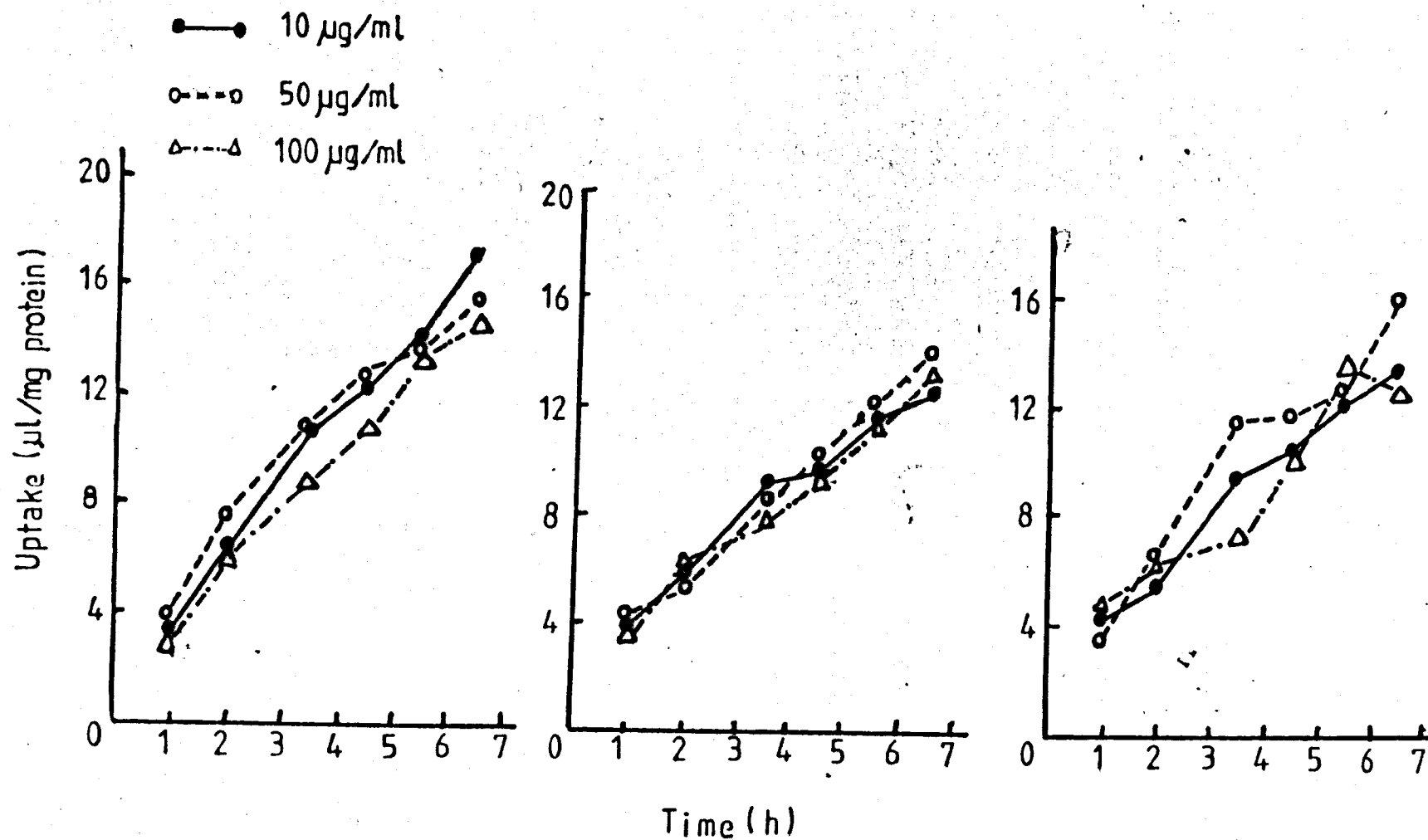


FIG.8A. EFFECT OF DIVEMA DERIVATIVES ON THE UPTAKE OF ^{125}I -PVP



uptake values are expressed as absolute Endocytic Indices, percentages of the matched control containing 0.02% bicarbonate and also as a percentage of the mean Endocytic Index obtained for the no serum uptake of ^{125}I -PVP in experiments carried out in Chapter 6. Figs 8.5. and 8.6. show the effect of unmodified and modified DIVEMAs on the uptake of ^{125}I -PVP in the absence of serum.

8.2.2. Preliminary Experiments to show the effect of DIVEMAs and DIVEMA Derivatives on the uptake of colloidal ^{198}Au .

Preliminary experiments undertaken to examine the effect of DIVEMAs and their derivatives on the uptake of colloidal ^{198}Au are shown in Table 8.5. and Fig. 8.7. and 8.8. Although none of the substances had any appreciable stimulatory effect, the molecule containing 10 - 14% pyrrolidone appeared to be somewhat inhibitory. It is apparent from the correlation coefficients calculated for the individual experiments and from Figs. 8.7. and 8.8. that the results obtained were not conclusive.

TABLE 8.4. EFFECT OF DIVEMAS ON THE UPTAKE OF 125 I-PVP IN THE
ABSENCE OF SERUM

| DIVEMA (50 μ g/ml) | Endocytic Index | Corr | Control Endocytic Index | % Control | Mean Percentage Matched Control | Mean Endocytic Index |
|---------------------------|--------------------|-------|-------------------------------|-----------|--|----------------------------|
| S9 | 0.69 | 0.646 | 0.56 | 123 | | |
| | 1.95 | 0.921 | 1.25 | 156 | 120% | 1.50 \pm 0.41 |
| | 1.87 | 0.696 | 2.29 | 82 | | |
| S11 | 2.21 | 0.935 | 0.56 | 176 | | |
| | 2.82 | 0.849 | 1.25 | 226 | 172% | 2.55 \pm 0.18 |
| | 2.62 | 0.889 | 2.29 | 114 | | |
| S12 | 1.38 | 0.782 | 0.56 | 246 | | |
| | 7.15 | 0.853 | 1.25 | 572 | | |
| | 2.37 | 0.904 | 2.29 | 104 | 252% | 2.78 \pm 1.01 |
| | 1.19 | 0.879 | 0.99 | 120 | | |
| | 1.79 | 0.826 | 0.99 | 217 | | |
| S14 | 1.08 | 0.513 | 0.56 | 193 | | |
| | 1.43 | 0.939 | 1.25 | 114 | 126% | 1.39 \pm 0.16 |
| | 1.65 | 0.838 | 2.29 | 72 | | |

FIG.8.5. EFFECT OF DIVEMA ON THE UPTAKE OF 125 I-PVP IN ABSENCE OF SERUM

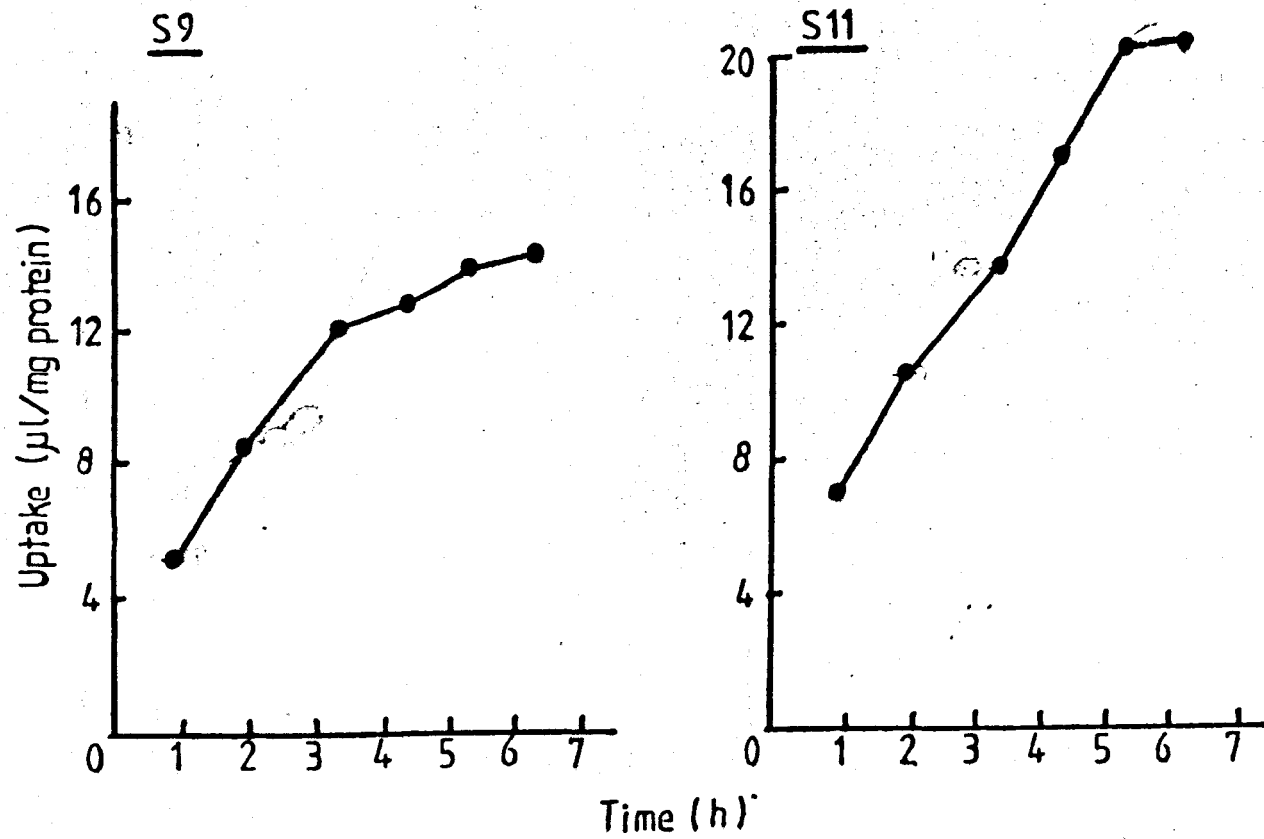


FIG.8.6. EFFECT OF MODIFIED DIVEMA ON THE UPTAKE OF ^{125}I -PVP IN THE ABSENCE OF SERUM

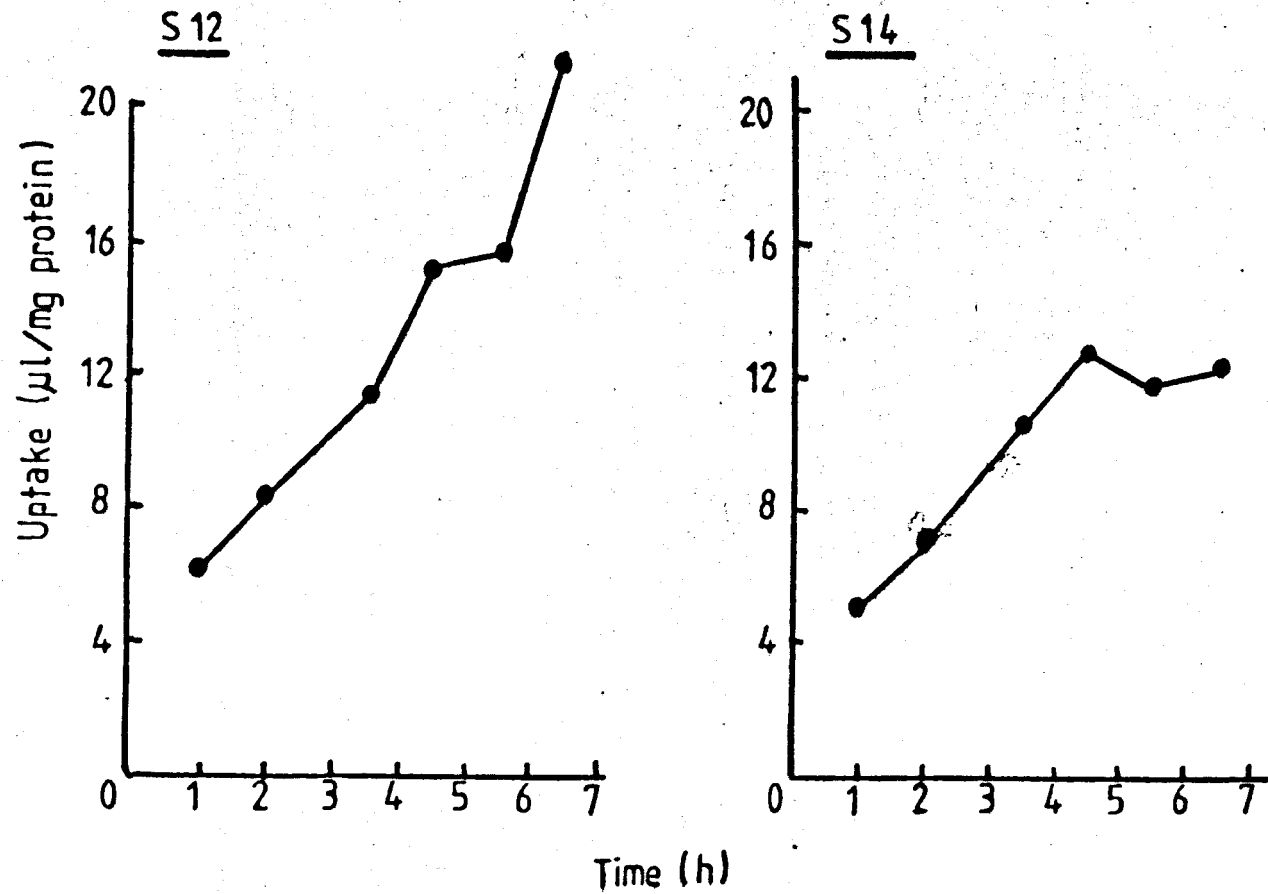


TABLE 8.5. UPTAKE OF COLLOIDAL ^{198}Au IN THE PRESENCE OF DIVEMAS

| DIVEMA (50 $\mu\text{g}/\text{ml}$) | Endocytic Index | Corr. Coeff. | Mean Endocytic Index (\pm SE) | Matched Control (\pm NaHCO ₂) Endocytic Index | Corr. Coeff. |
|---|--------------------|-----------------|--|--|-----------------|
| S9 | 3.25 | 0.950 | | 1.6 | 0.915 |
| | 4.98 | 0.579 | 3.13 \pm 1.11 | 5.35 | 0.882 |
| | 1.15 | 0.624 | | 4.34 | 0.932 |
| S11 | 2.35 | 0.715 | 2.51 | 1.6 | 0.915 |
| | 2.67 | 0.787 | | 5.35 | 0.882 |
| S12 | 3.36 | 0.976 | | 1.6 | 0.915 |
| | 3.55 | 0.981 | 2.75 \pm 0.70 | 3.43 | 0.928 |
| | 1.35 | 0.766 | | 4.34 | 0.932 |
| S13 | 2.57 | 0.912 | | 2.21 | 0.901 |
| | 0.74 | 0.777 | 2.22 \pm 0.77 | 2.21 | 0.901 |
| | 3.35 | 0.928 | | 2.21 | 0.901 |
| S14 | 2.72 | 0.970 | | 1.6 | 0.915 |
| | 1.19 | 0.785 | 1.75 \pm 0.49 | 4.34 | 0.932 |
| | 1.35 | 0.766 | | 4.34 | 0.932 |

Overall Mean (\pm SE) Control
Endocytic Index

3.39 \pm 0.62

FIG. 8.7. EFFECT OF DIVEMA ON THE UPTAKE OF COLLOIDAL ^{198}Au

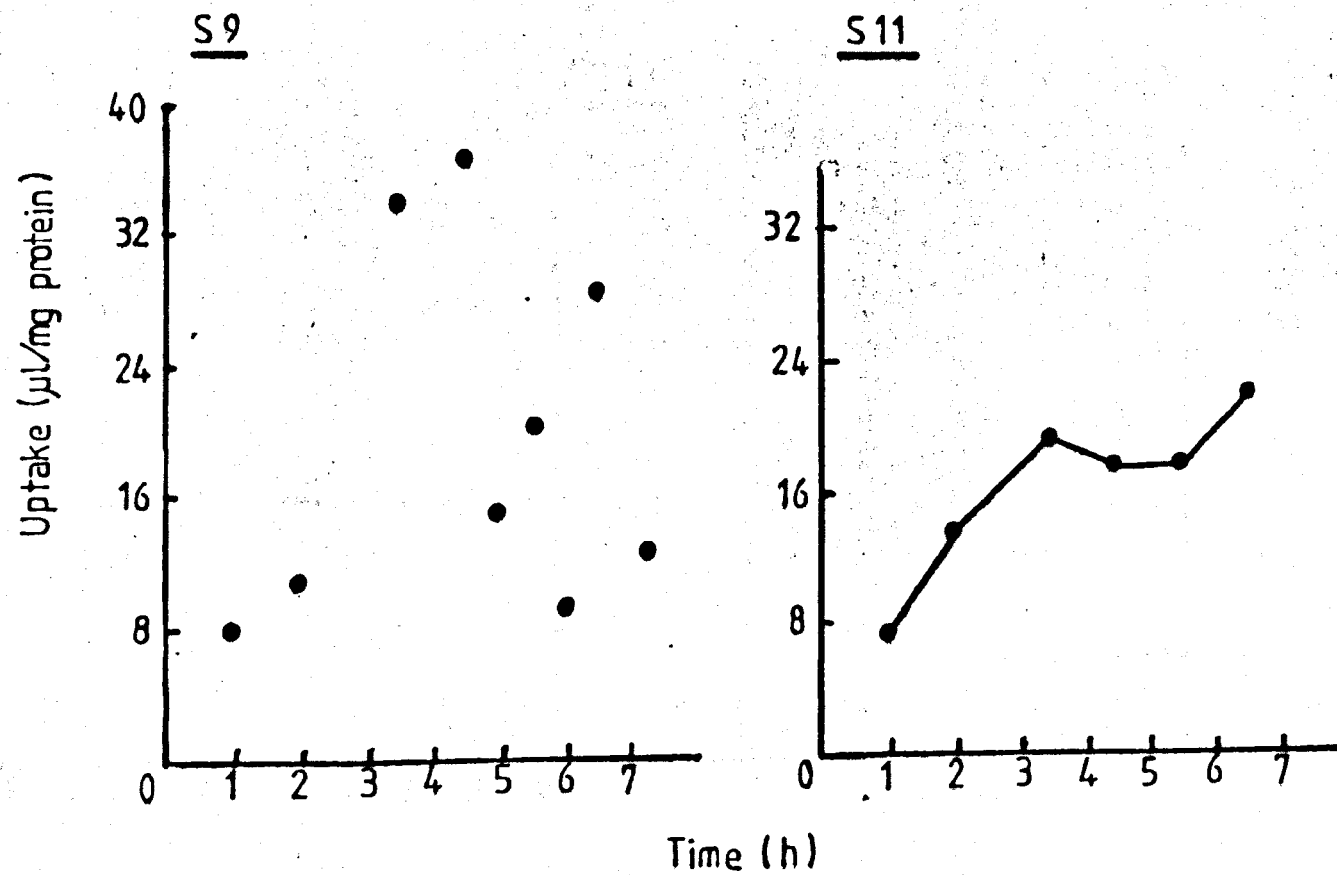
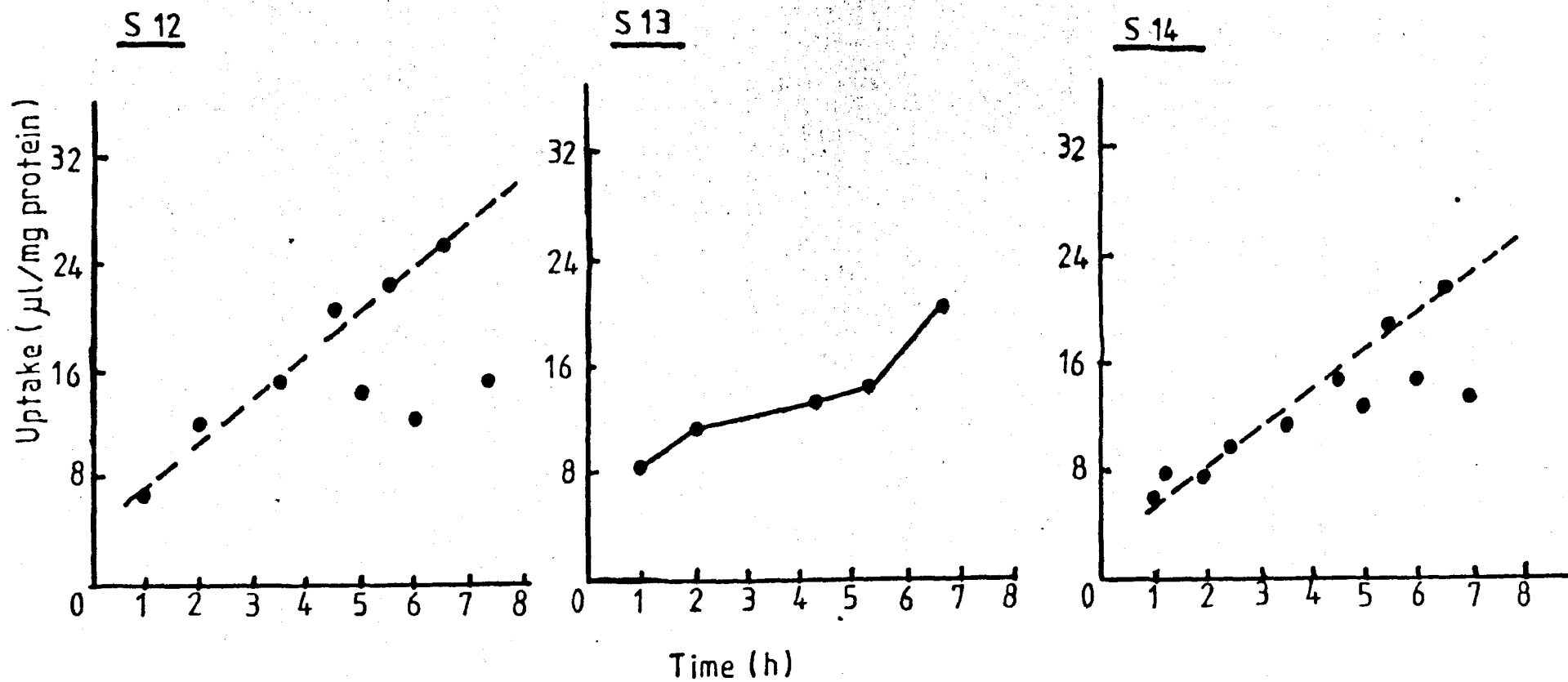


FIG.8.8.EFFECT OF MODIFIED DIVEMA ON THE UPTAKE OF COLLOIDAL ^{198}Au



8.3. DISCUSSION

None of the DIVEMAs examined had any significant effect on the uptake of ^{125}I -PVP. The smallest unmodified DIVEMA (S9) seemed to affect the uptake of ^{125}I -PVP to the greatest extent and this was most apparent when the data were expressed as a percentage of their matched control. When S9 was present at a concentration of 10 $\mu\text{g}/\text{ml}$, the Endocytic Indices for ^{125}I -PVP gave a mean value of 75% when expressed as a percentage of their matched controls, whereas a DIVEMA concentration of 100 $\mu\text{g}/\text{ml}$ the mean percentage was 124%. This could have been a trend towards slight inhibition at low concentration and slight stimulation at high concentration, but the responses, if real, were only marginal. Throughout all the experiments using ^{125}I -PVP as a marker, tissue accumulation retained a linearity with time, there was no evidence for toxicity of DIVEMA even at the highest concentration. A single experiment was carried out with 0.1% sodium bicarbonate in the culture medium just to confirm that the usual concentration of 0.02% or less (depending on the quantity used up during hydrolysis of the DIVEMA) could not affect the pinocytic uptake of ^{125}I -PVP. The Endocytic Index measured in the presence of 0.1% sodium bicarbonate (pH 7.8) was 1.62 $\mu\text{l}/\text{mg}/\text{protein}/\text{h}$ and this was well within the usual range for this substrate (see Chapter 3).

In case the calf serum in the culture medium was interacting with DIVEMA in some way and interfering with its biological activity, cultures were performed under serum-free conditions. Omission of calf serum from the culture system served to reveal sodium bicarbonate inhibition of ^{125}I -PVP uptake, which was most marked in the experiments not containing DIVEMA, where the concentration of bicarbonate would

be at its highest. Addition of DIVEMA (50 $\mu\text{g/ml}$) would lower the bicarbonate concentration and the Endocytic Indices for ^{125}I -PVP measured in the presence of these substances were more comparable with the usual Endocytic Indices obtained for the rate of uptake of ^{125}I -PVP in the absence of serum ($2.94 \pm 0.37 \mu\text{l/mg protein/h}$) see Chapter 6. Although uptake was linear with time in some experiments, others showed more scatter than would be desirable, but this is often the case when serum is not included in the culture system (Ibbotson & Williams, 1979). The average uptake graphs indicate that the high molecular weight DIVEMA (S11) and the betaine-substituted DIVEMA (S14) did not have any effect on the progressive accumulation of ^{125}I -PVP, whereas both the low molecular weight DIVEMA (S9) and the pyrrolidone-substituted species (S12) may have caused some degree of inhibition after 3.5 - 5h. It has been suggested that no serum cultures are not reliable after 5h (Ibbotson, 1978), so this could mean that the inhibitory trends were simply a reflection of serum dependence. However, conclusion in this direction would be difficult as it would imply that the other two DIVEMAs were in some way stimulating pinocytosis to maintain linearity and thus compensate for any deleterious effect caused by the absence of serum. Schnee (unpublished observation) has shown that DIVEMA precipitates in medium 199 at high concentration (400 $\mu\text{g/ml}$) when serum is removed. There was no evidence for this effect at the lower concentrations employed.

Dimethyl formamide alone had a very marked inhibitory effect on the uptake of ^{125}I -PVP in the absence of serum so it was impossible to study the action of the hydrophobic DIVEMA in this system.

Having assessed the effect of DIVEMA on fluid-phase pinocytosis it was considered important to investigate the effect on a pinocytic

marker that is known to interact with the plasma membrane.

Colloidal ^{198}Au was chosen for this purpose as α -amino acids produced such interesting modifications of its rate of capture (Chapter 5). Unfortunately time prevented the completion of this study, so only preliminary results are stated here and it is emphasized that these data need expanding.

According to the estimated Endocytic Indices the only polymer to have any marked effect on the uptake of colloidal ^{198}Au was the pyrrolidone-substituted DIVEMA which produced a mean value of $1.75 \pm 0.49 \mu\text{l/mg protein/h}$, in percentage terms the mean being 76.1% the control. However, throughout these experiments the frequent but apparently random appearance of data with excessive scatter of uptake points was rife. Examination of the average uptake graphs probably reflect more meaningful trends. The betaine and pyrrolidone-substituted substances did not modify pinocytosis of colloidal ^{198}Au appreciably, there being good linear correlation between most of the points taken at differing sample times so they could not be incorporated easily into the overall mean values. These discrepant values can probably be disregarded. The hydrophobic DIVEMA (S13) caused some inhibition in the rate of accumulation of colloidal ^{198}Au but did not disturb the linearity of uptake with time, whereas the high molecular weight unmodified DIVEMA (S11) caused no effect during the first 3.5h but inhibited progressive accumulation totally after this time. The results obtained with the low molecular weight DIVEMA (S9) were very variable. The average graph shows more or less random scatter of points and for this reason no attempt is made to describe the relationship between them.

From an overview of all the results obtained with colloidal ^{198}Au it is possible to draw tentative conclusions. There was certainly no stimulation of pinocytosis of colloidal ^{198}Au , in agreement with the data obtained using ^{125}I -PVP, but in some cases there was marked inhibition. It is possible that the DIVEMA is itself interiorized in such a way that it competes for membrane receptor sites normally occupied by colloidal ^{198}Au and this would explain the lower Endocytic Indices for colloidal ^{198}Au seen in the presence of the hydrophobic species. The effect observed with the largest DIVEMA (S11) is not readily explicable using this approach.

Although the experimental data obtained here (indicating that neither DIVEMA nor its derivatives enhance pinocytic rate) do not reinforce the earlier observations of Breslow et al., (1973), it is possible that the differences seen relate to the endocytic capacities of the cells involved. Rat yolk sacs are restricted to pinocytic activity, whereas blood clearance of particulate substrate is usually interpreted as an indication of the phagocytic activity of cells of the reticuloendothelial system. Maybe DIVEMA can only potentiate the phagocytic mechanism. An alternative explanation for the discrepancy could be the differences in methodology employed. It is now widely agreed that extrapolation from blood clearance to phagocytic rate has many hazards and these are discussed extensively by Pratten, et al., (1979). Even in an original report relating to DIVEMA and its effect on phagocytosis, (Munson et al., 1970) the weaknesses of the system that was used were acknowledged. An interesting point to consider here is the possible loss of low molecular weight DIVEMA through the kidney glomerulus. The marked stimulatory effect reported for the lower molecular weight DIVEMAS in vivo is

difficult to reconcile with their transient presence in the bloodstream. However, perhaps the interaction of even a small quantity of DIVEMA with the reticuloendothelial system may be sufficient to have a profound effect, or conversely the DIVEMA itself may interact with serum proteins and remain in the circulation for considerably longer than one might expect. Assuming the stimulation of blood clearance observed by Breslow et al., (1973) was a "real" effect on endocytosis, it would be interesting to examine this observation further using a more quantitative in vitro measure of phagocytic rate and determine if the polymer simply enhances substrate binding or actually stimulates membrane internalization.

Recently a study has been made by M.K. Pratten in this laboratory to assess the effect of DIVEMAs and DIVEMA derivatives on pinocytosis in the rat peritoneal macrophage and she used exactly the same substances employed in the above experiments with the rat visceral yolk sac. It was found that none of polymers (S9 - S14) had any stimulatory effect on the pinocytic uptake of ^{125}I -PVP and colloidal ^{198}Au . This was particularly interesting as DIVEMA is known to activate macrophages (Kaplan et al., 1974) a phenomenon which is believed to cause enhancement of certain phagocytic events (Hamburg et al., 1978) and has tenuously been linked with enhanced pinocytic activity. Activation of macrophages usually takes approximately 72h for completion, so Pratten also pre-incubated macrophages in DIVEMA before monitoring pinocytic rate. Although the total exposure time was up to 100h the pyran still had no stimulatory effect.

DIVEMA itself is potentially a very interesting compound due to its wide variety of biological activity and also due to its usefulness as a drug carrier. It would be very interesting to extend this investigation to include experiments which monitor the pinocytosis of DIVEMA itself and in the near future this should be possible as we hope to acquire a [^{14}C] DIVEMA sample. Perhaps the rate of pinocytosis of DIVEMA might be the key to resolving the mechanism of some biological effects. A high rate of capture could ensure a high intracellular level of substance and conceivably this may be a prerequisite for an internal trigger necessary to induce the observed biological responses. Although a low rate of capture would not exclude action via an intracellular mediator, it could support the hypothesis that DIVEMA exerts its effect entirely by an interaction with the plasma membrane of the target cells.

CHAPTER 9

PREPARATION OF ^{125}I -PVP

9.1. INTRODUCTION

Much of the work reported in this thesis involves the use of ^{125}I -PVP preparations purchased from the Radiochemical Centre, Amersham. The specifications of this product and its behaviour in the rat visceral yolk sac system as a marker of fluid-phase pinocytosis are described in Chapter 3. The development of a technique for the production of ^{125}I -PVP within the laboratory was not only an interesting exercise, but was necessary to enable the radiolabelling of a number of different molecular weight fractions of PVP kindly donated by Professor H. Ringsdorf, University of Mainz. The rates of pinocytosis of these compounds is discussed later (Chapter 10).

Although the nature of, and the chemical reactions involved in, the coupling of $[^{125}\text{I}]$ iodide to PVP remain obscure, there are several methods described in the literature for the preparation of ^{125}I -PVP (Ravin et al., 1952; Gordon, 1958; Regoeczi, 1976). Here the technique of Regoeczi (1976) was used for labelling PVP (a method that corresponds to a scaled down version of the one suggested by the Radiochemical Centre). Various methods were investigated to assess their potential for the routine removal of excess $[^{125}\text{I}]$ iodide from the ^{125}I -PVP preparations produced. These included anion-exchange chromatography, Sephadex column chromatography and dialysis. The stability of the ^{125}I -PVPs produced was estimated both during storage and under culture conditions and, to complete the investigation, the effect of $[^{125}\text{I}]$ iodide on the pinocytosis of ^{125}I -PVP was also examined.

9.2. METHODS

9.2.1. Iodination of PVP

The method of Regoeczi (1976) was used to form ^{125}I -PVP. PVP (100mg) was dissolved in 1ml of 2.0M sulphuric acid at 0°C and placed in a small quartz test-tube. Then 0.1ml of sodium nitrite (10% w/v) was added followed by 0.5 - 2.0 mCi of sodium ^{125}I iodide. The mixture was then irradiated by an ultraviolet source (Anovia, medium pressure) for 1h in a stream of cold air, the lamp being left to warm up for 15min beforehand. The solution was neutralized with 2.0ml of 0.2M potassium hydroxide and reduced by the addition of 10mg of sodium sulphite in 0.2ml of distilled water.

9.2.2. Separation of ^{125}I iodide from ^{125}I -PVP

A paper electrophoresis separation technique was used to assess the percentage ^{125}I iodide within the ^{125}I -PVP preparations at any time (see Section 2.8.). Routinely, a sample of the reaction mixture was taken immediately following the radiolabelling procedure and the percentage ^{125}I iodide remaining was measured. The labelling efficiency could then be calculated.

Samples taken from the reaction mixtures were then subjected to anion-exchange chromatography (see Section 2.10.2.) or Sephadex column chromatography (see Section 2.10.1.), or alternatively they were dialysed against 1% sodium chloride for three days with two changes of sodium chloride per day. Following these treatments various samples were taken and subjected to electrophoresis to determine the percentage ^{125}I iodide remaining.

In some cases the percentage ^{125}I iodide in the treated preparations of ^{125}I -PVP was assessed at various times after treatment to determine whether any decomposition of the polymer complex was occurring. Following dialysis a small portion of each ^{125}I -PVP preparation was incubated for 6h at 37°C in a shaking water bath and the percentage ^{125}I iodide in

each preparation estimated both before and after the incubation, to give an estimation of the stability of the preparation during culture.

9.2.3. PVP samples used

Four samples of PVP with different molecular weight distributions (Fig.9.1.) were subjected to the radioiodination procedure. They will be referred to as follows:

| <u>Code</u> | <u>Mean Molecular Weight</u> |
|-------------|------------------------------|
| S1 | 2,500 |
| S2 | 50,000 |
| S3 | 84,000 |
| S4 | 700,000 |

All were subsequently dialysed, but not all the ^{125}I -PVPs were subjected to each type of chromatography.

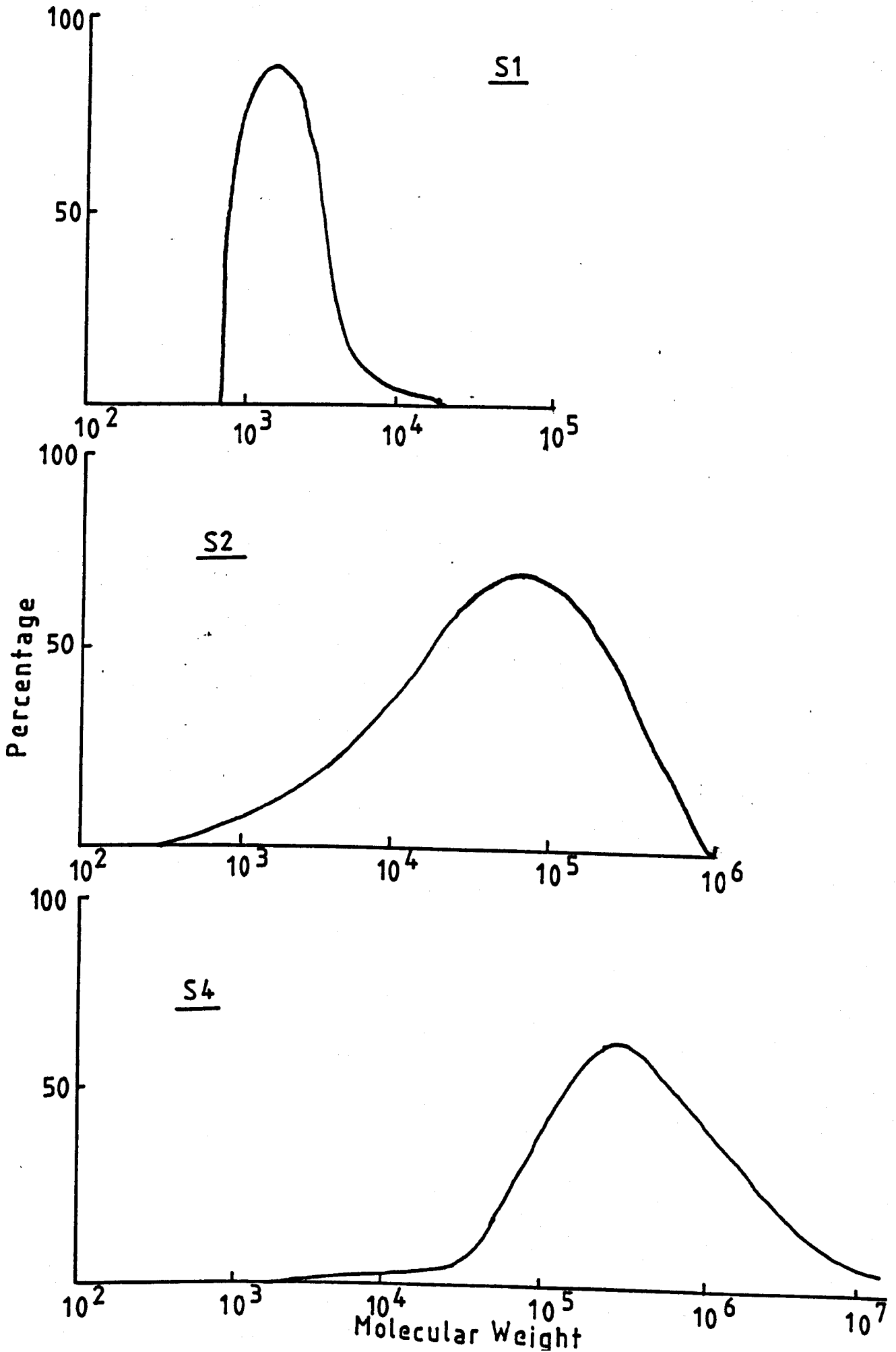
9.2.4. Pinocytosis of ^{125}I -PVP preparations containing a substantial quantity of ^{125}I iodide by the rat visceral yolk sac

For this investigation mixtures were used containing ^{125}I -PVP and ^{125}I iodide, both purchased from the Radiochemical Centre. It was difficult to add exactly the same concentration of ^{125}I iodide to each stock solution of ^{125}I -PVP, so the percentage ^{125}I iodide in the mixture varied slightly. Incubations were set up containing the following radiotracers:

1. ^{125}I -PVP at known concentration
2. ^{125}I iodide at an unknown concentration
3. ^{125}I -PVP / ^{125}I iodide mixture containing the same specific activity of the substrates used in 1. and 2.

The normal culture procedure was followed (see Chapter 2) and the accumulation of radioactivity measured in terms of $\mu\text{l}/\text{mg}$ protein. At the beginning of each experiment the percentage ^{125}I iodide in the mixture was estimated by electrophoresis. The uptake of ^{125}I -PVP from the ^{125}I -PVP / ^{125}I iodide mixture was quantitated by firstly taking the

FIG. 9.1. MOLECULAR WEIGHT DISTRIBUTIONS OF THREE
OF THE PVP SAMPLES



medium counts representing $[^{125}\text{I}]$ iodide activity from the medium total counts, thus giving a true value for the ^{125}I -PVP activity in the culture medium.

9.3. RESULTS

9.3.1. Electrophoresis as a suitable method for the estimation of the percentage $[^{125}\text{I}]$ iodide in a preparation of ^{125}I -PVP

When a sample of ^{125}I -PVP containing some $[^{125}\text{I}]$ iodide was subjected to electrophoresis the ^{125}I -PVP remained almost static at the point of application, whereas the $[^{125}\text{I}]$ iodide migrated towards the cathode. Fig.9.2. shows the migration of $[^{125}\text{I}]$ iodide with increasing time of electrophoresis and Fig.9.3. shows the rate of iodide movement with time. An electrophoresis time period of 35min was chosen for all subsequent studies.

9.3.2. Efficiency of labelling

Table 9.1. shows the efficiency of labelling of S1 to S4. In the first preparation S2 seemed to provide the optimum size distribution, it having the maximum efficiency of labelling (45.7%). As the molecular weight distributions deviated from that of S2 labelling efficiency fell, being approximately 20% at the extremes of the range. The second preparation showed slightly more variability, but again maximum labelling was achieved with S2 (43.9%).

Figs. 9.4. to 9.7. show the radioactivity patterns obtained after electrophoresis of the crude reaction mixtures of S1 to S4.

9.3.3. Removal of contaminating $[^{125}\text{I}]$ iodide from the crude reaction mixtures of ^{125}I -PVP

The method of Regoeczi (1976) suggested that unbound radioactivity could be efficiently removed from ^{125}I -PVP preparations by anion-exchange chromatography. Samples from the crude reaction mixtures of S1 and S3 were passed through an anion-exchange column and a typical elution profile is shown in Fig.9.8. Samples taken from the fractions containing the peak of radioactivity from the S1 and S3 anion-exchange runs were then subjected to electrophoresis. The radioactivity distributions obtained for these two samples are shown in Fig.9.9. and Fig.9.10.

FIG. 9.2. MIGRATION OF [125 I] IODIDE WHEN
SUBJECTED TO PAPER ELECTROPHORESIS

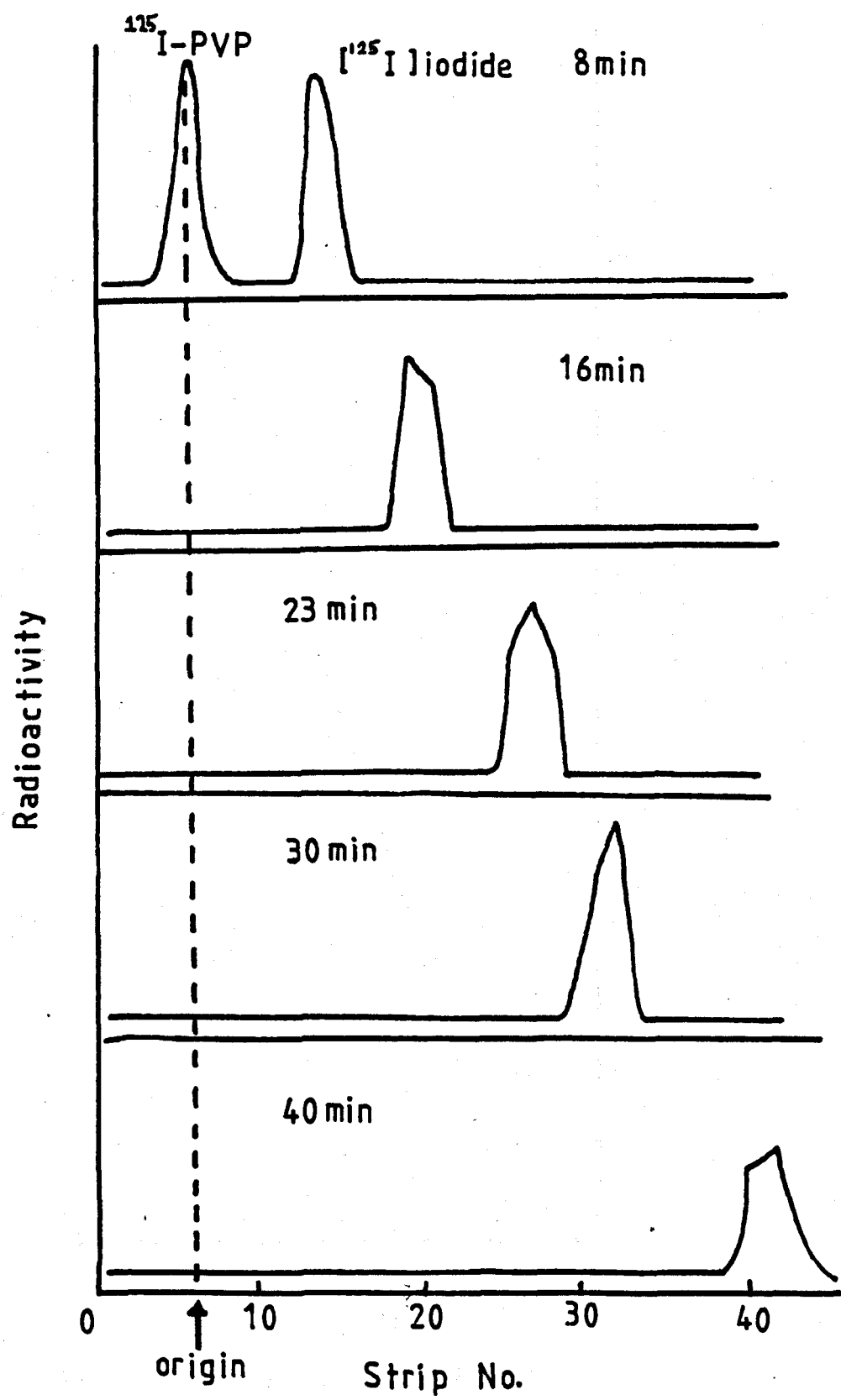


FIG. 9.3. MIGRATION OF [125 I] IODIDE WITH INCREASING ELECTROPHORESIS TIME

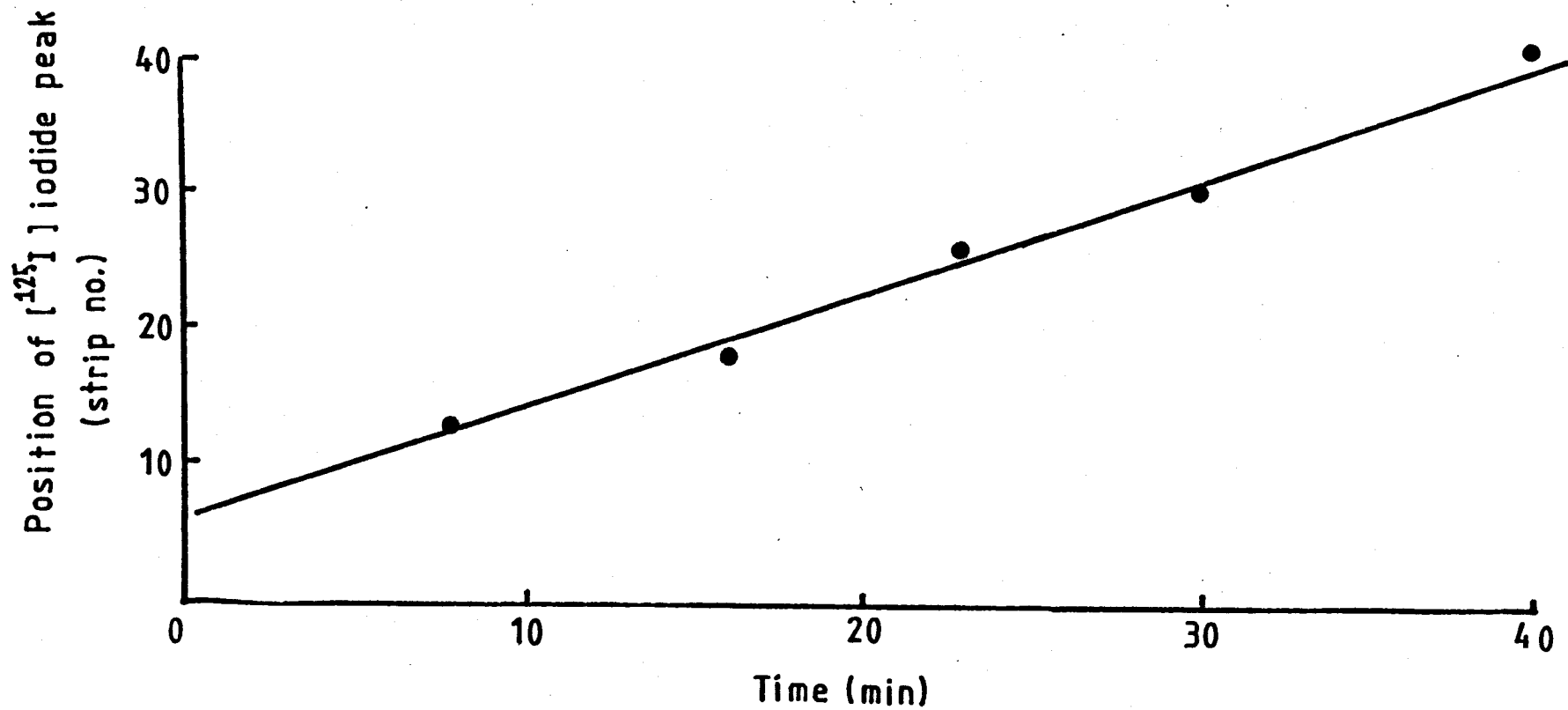


TABLE 9.1. EFFICIENCY OF LABELLING OF THE ^{125}I -PVPs S1, S2, S3, and S4

| <u>Code</u> | <u>Preparation 1</u> | <u>Preparation 2</u> |
|-------------|----------------------|----------------------|
| S1 | 21.4 | 32.7 |
| S2 | 45.7 | 43.9 |
| S3 | 33.4 | 19.9 |
| S4 | 25.0 | 30.1 |

Figures represent the percentage of [^{125}I]iodide administered that became attached to PVP.

FIG. 9.4. ELECTROPHORESIS OF S1 REACTION MIXTURE

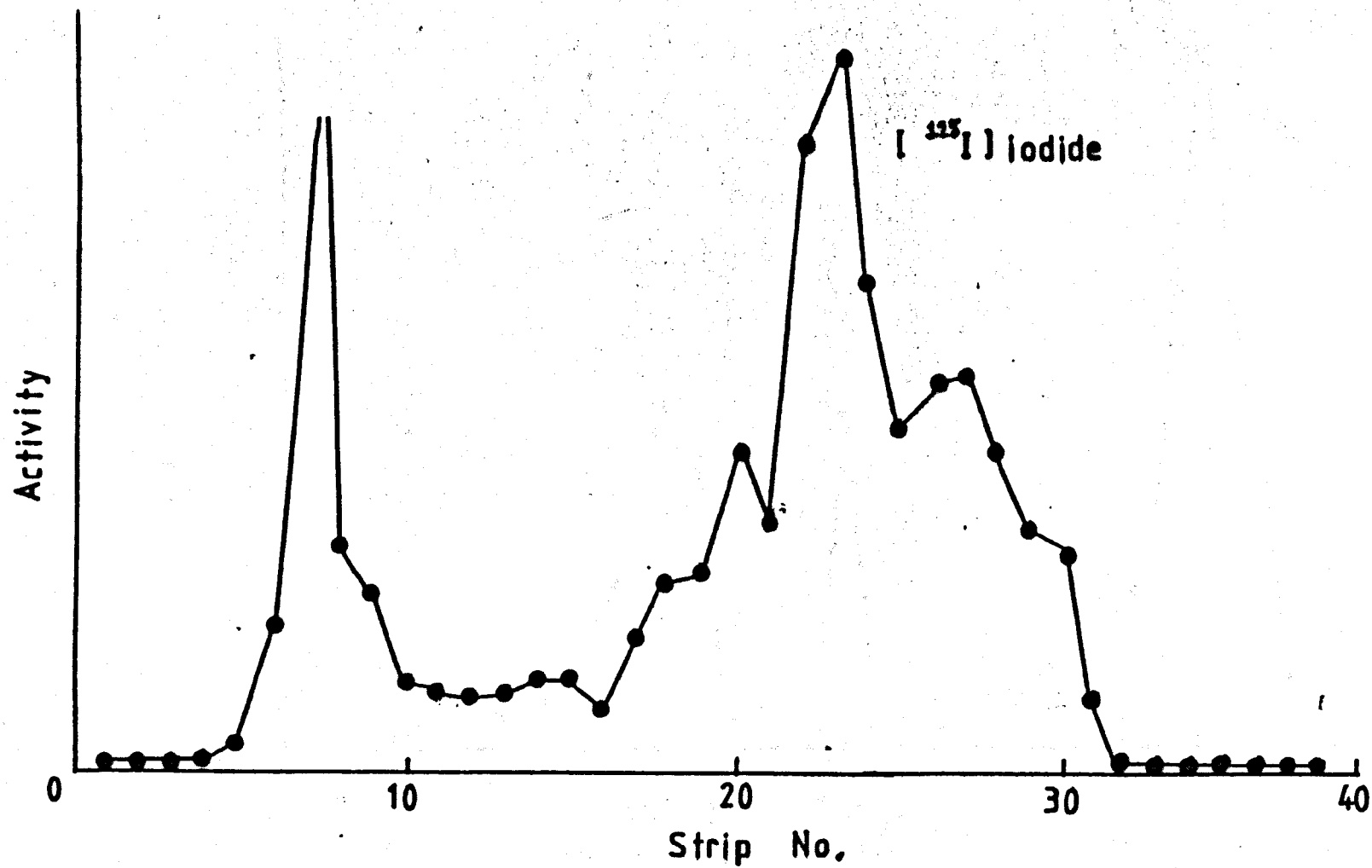


FIG. 9.5. ELECTROPHORESIS OF S2 REACTION MIXTURE

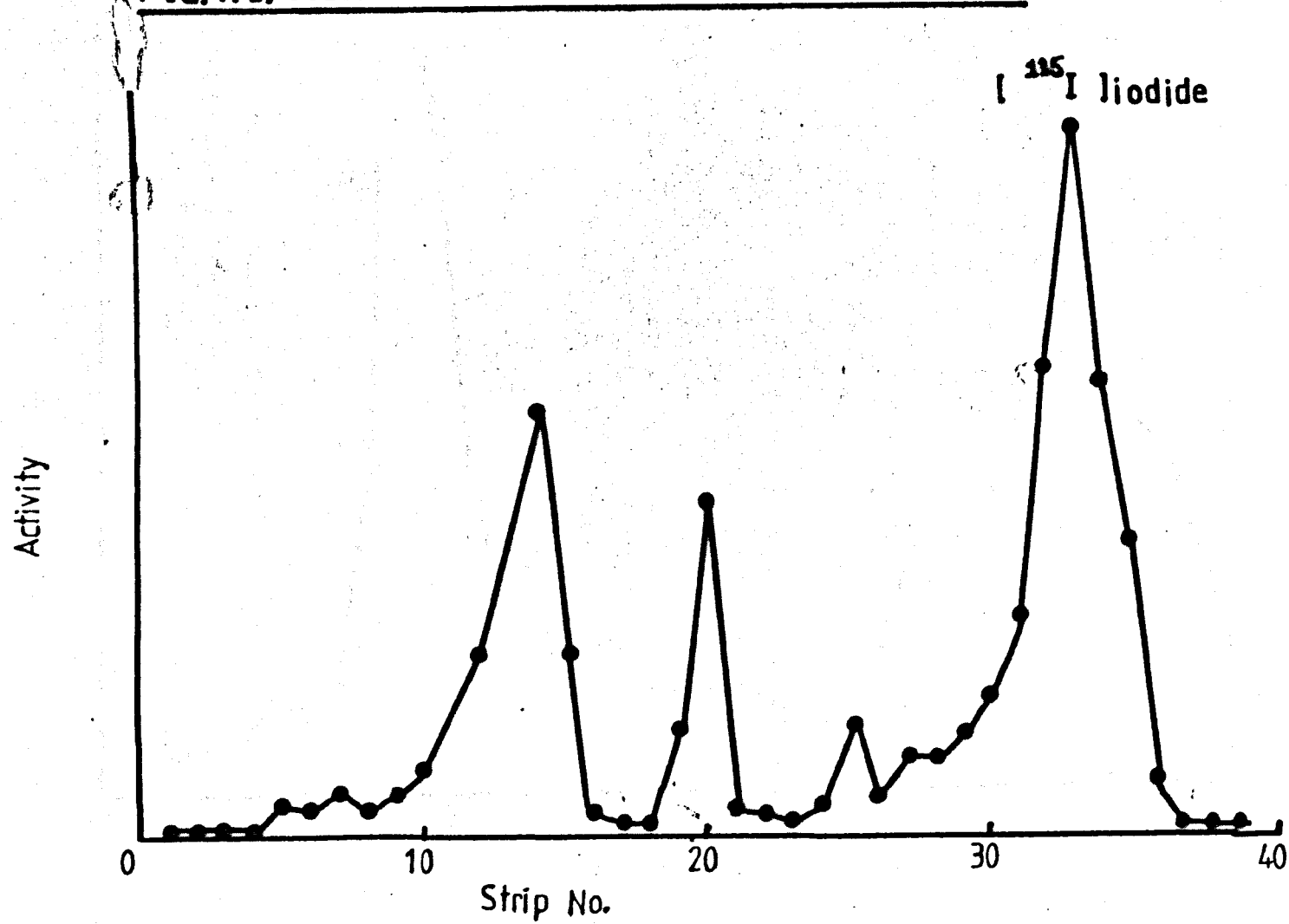


FIG. 9.6. ELECTROPHORESIS OF S3 REACTION MIXTURE

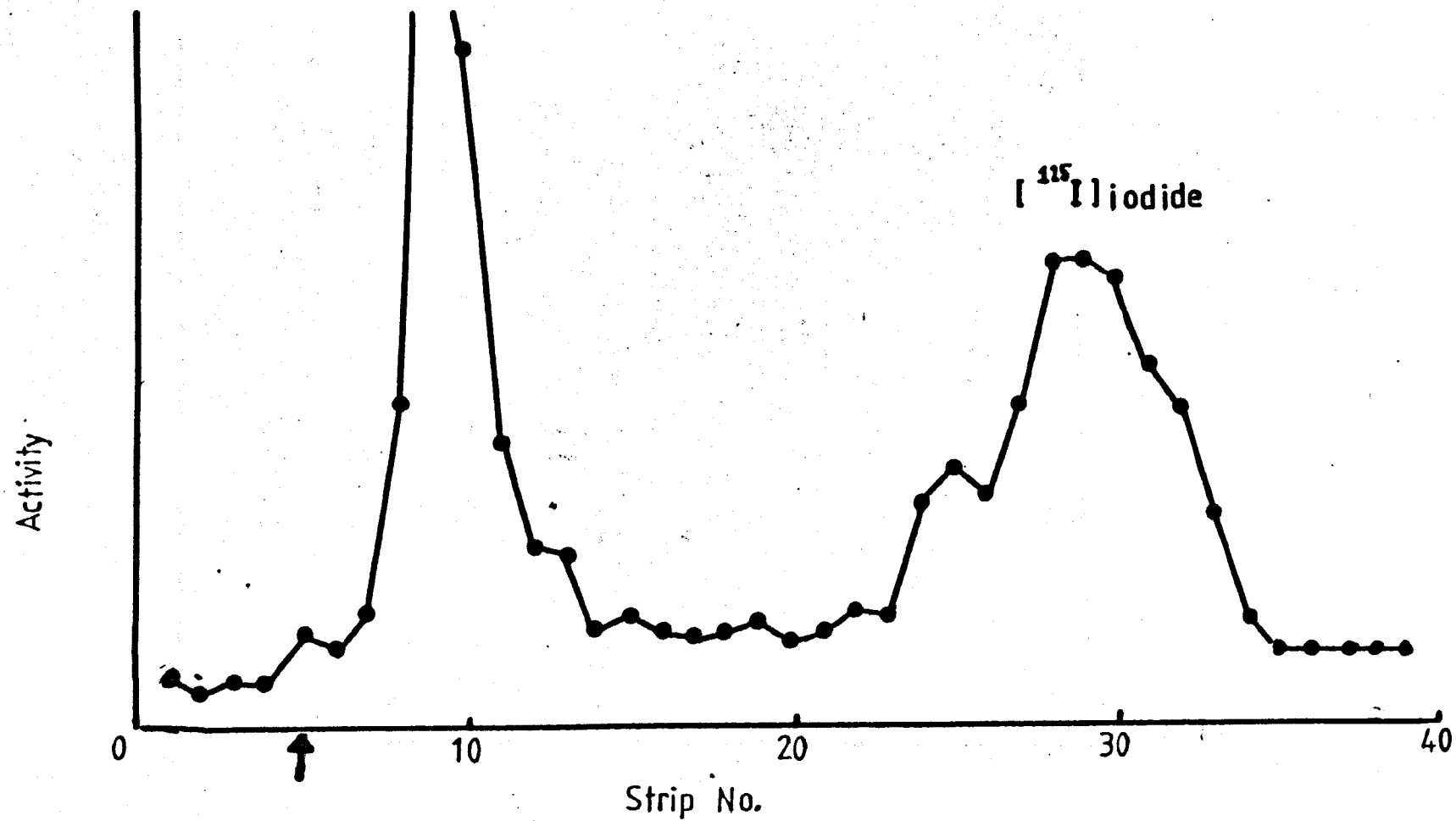


FIG. 9.7. ELECTROPHORESIS OF S4 REACTION MIXTURE

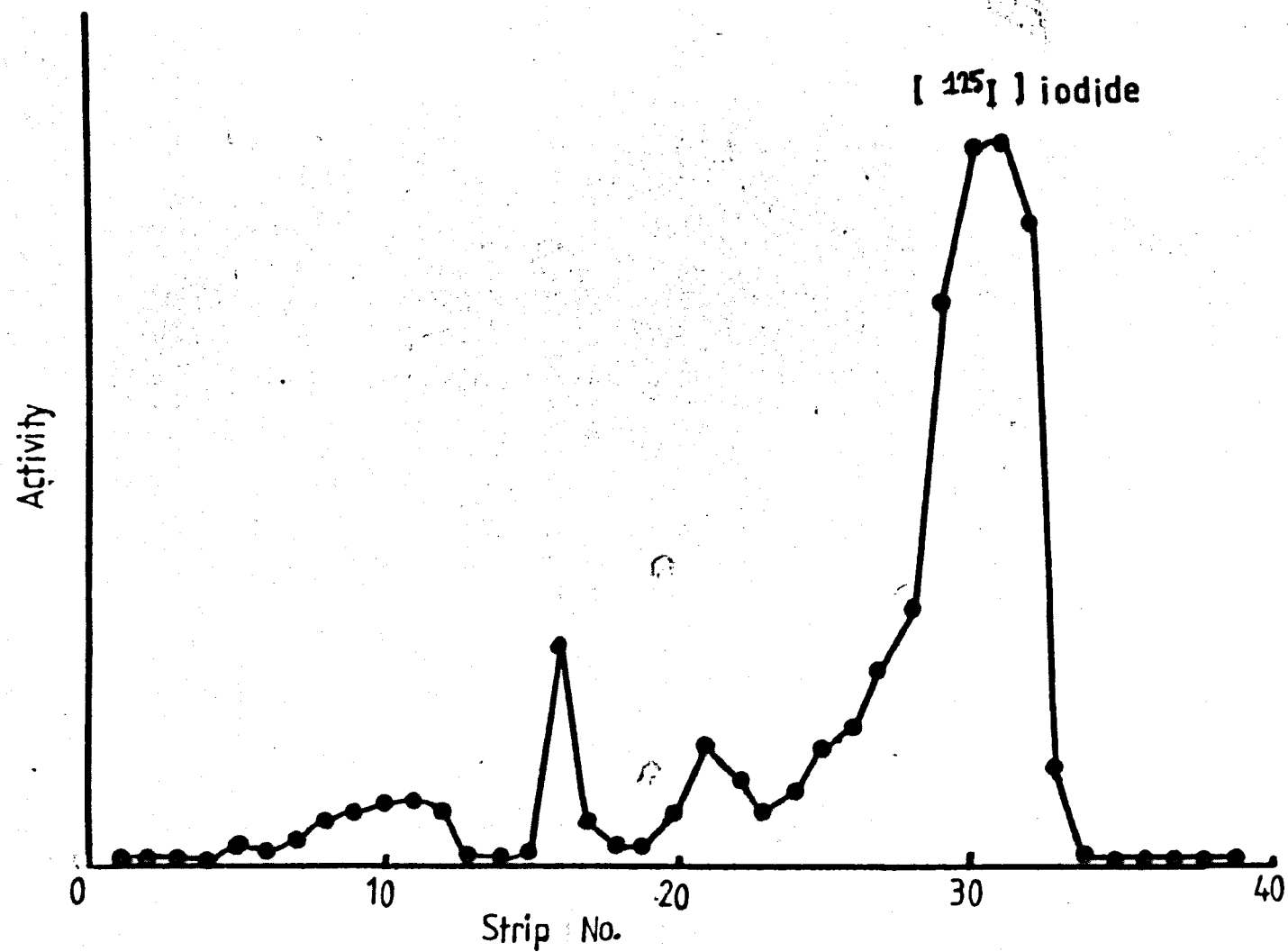


FIG.9.8. A TYPICAL ANION-EXCHANGE ELUTION PROFILE [S1]

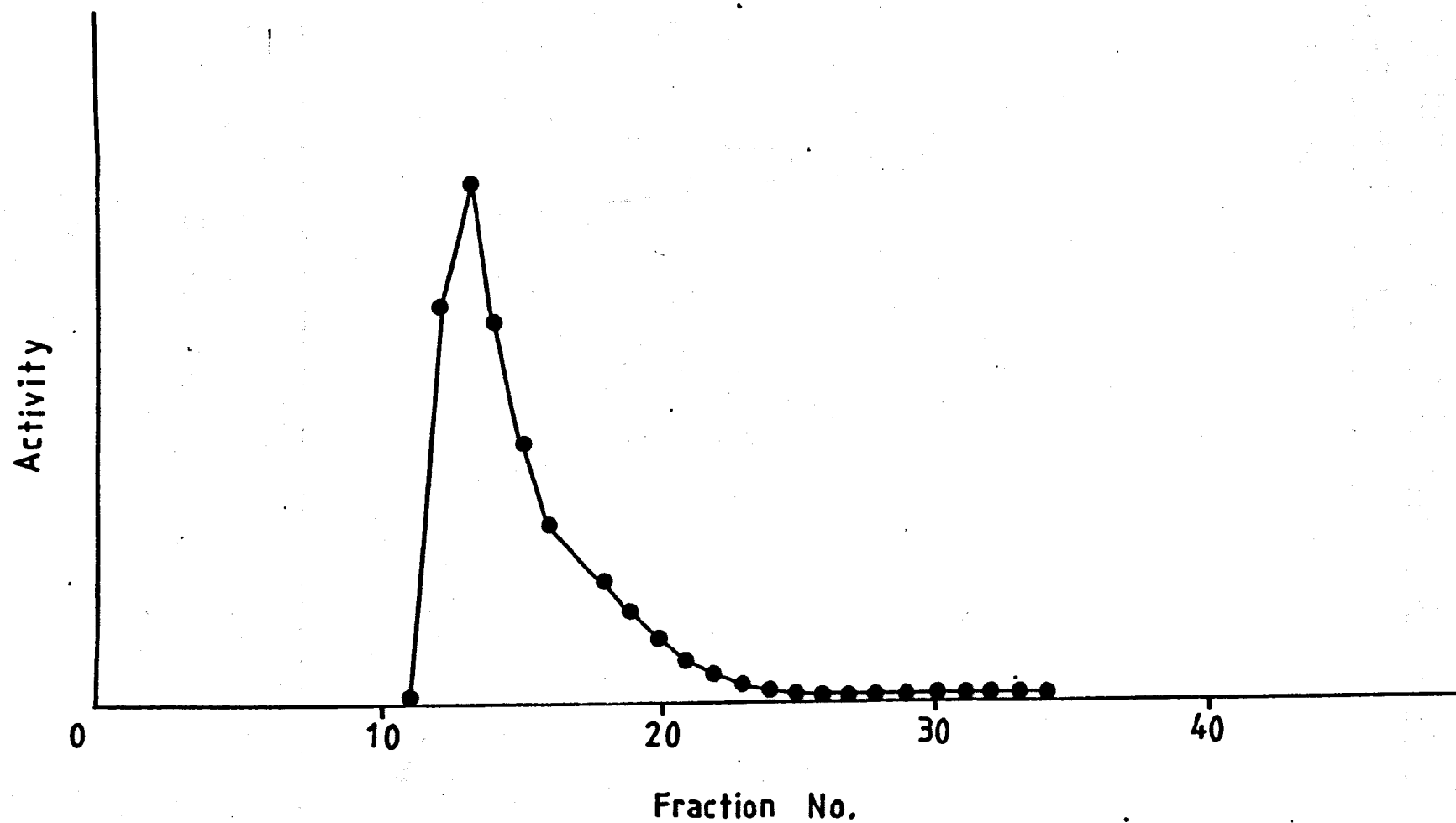


FIG.9.9.ELECTROPHORESIS OF S1 [Fraction 13] AFTER
ANION-EXCHANGE SEPARATION

(See Fig.9.8.)

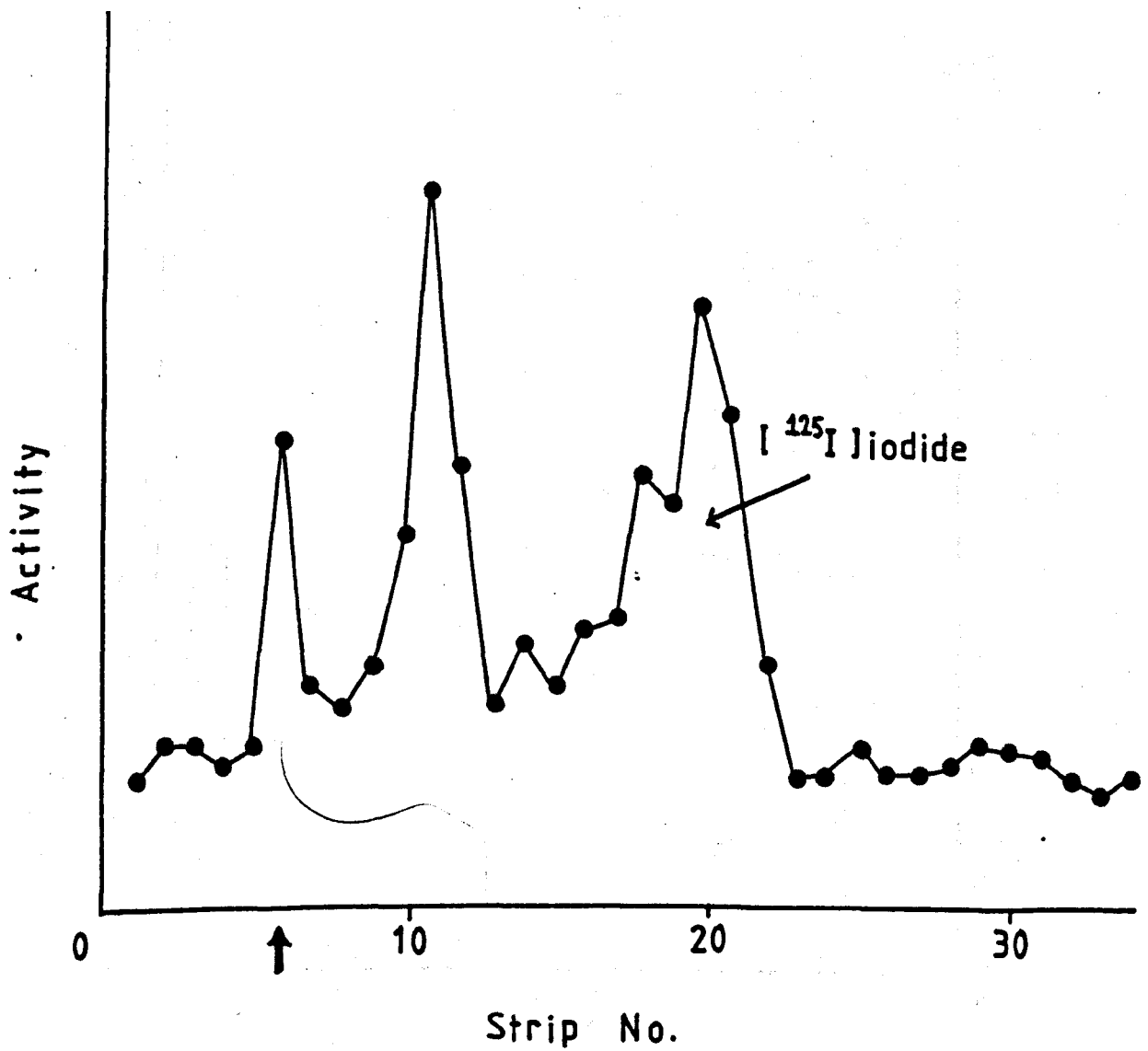
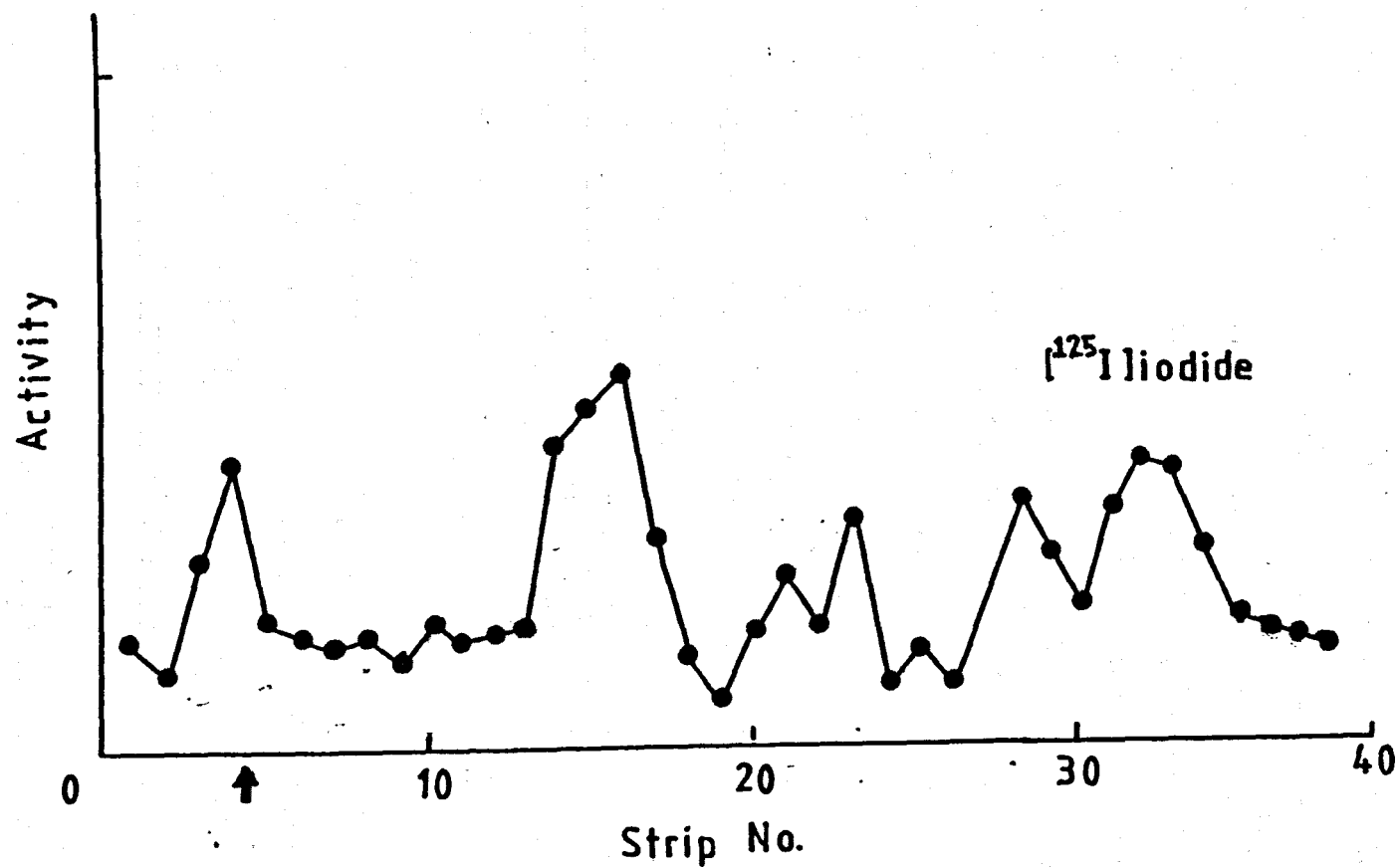


FIG. 9.10. ELECTROPHORESIS OF S3 FOLLOWING ANION-EXCHANGE
SEPARATION



respectively. It can be seen that in both cases there is still a considerable percentage of $[^{125}\text{I}]$ iodide in the preparations.

Figs. 9.11. and 9.12. show the Sephadex G-25 and G-10 separation of samples of the crude reaction mixture of S1. There is obviously a fairly large quantity of very low molecular weight ^{125}I -PVP. Samples taken from the void volume fractions, i.e. those furthest from the iodide peak, were run on paper electrophoresis and Figs. 9.13. and 9.14. show the radioactivity patterns produced. Again a high percentage of $[^{125}\text{I}]$ iodide was still present. Samples of the crude reaction mixtures of S3 and S4 were also put through a G-25 column and the elution profiles are shown (Figs. 9.15. and 9.16.). The electrophoresis pattern obtained from a sample of S3 taken from a fraction of the G-25 separation is shown in Fig. 9.17., again $[^{125}\text{I}]$ iodide is noticeably present. Samples taken from two fractions of the S4 G-25 elution were electrophoresed, the results obtained are shown in Figs. 9.18. and 9.19.

Following dialysis small aliquots of S1 to S4 were all subjected to electrophoresis (Figs. 9.20. to 9.23.).

9.3.4. Stability of ^{125}I -PVP preparations during storage and incubation

Table 9.2. shows the percentage $[^{125}\text{I}]$ iodide in preparations of ^{125}I -PVP at various times after dialysis, and Fig. 9.24. shows the rate of $[^{125}\text{I}]$ iodide release from each polymer.

Table 9.3. shows the percentage $[^{125}\text{I}]$ iodide in preparations of ^{125}I -PVP both before and following incubation at 37°C for 6h. There is not a dramatic increase in the free $[^{125}\text{I}]$ iodide content during this time.

9.3.5. Uptake of ^{125}I -PVP by the rat visceral yolk sac in the presence of $[^{125}\text{I}]$ iodide

Fig. 9.25. shows the accumulation of $[^{125}\text{I}]$ iodide measured in three different experiments. It can be seen that the quantity of $[^{125}\text{I}]$ iodide associated with the yolk sac was constant throughout the incubation. Table 9.4. shows the Endocytic Index of ^{125}I -PVP when measured in the presence or absence of large quantities of $[^{125}\text{I}]$ iodide.

FIG. 9.11. SEPHADEX G-25 SEPARATION OF S1 REACTION MIXTURE

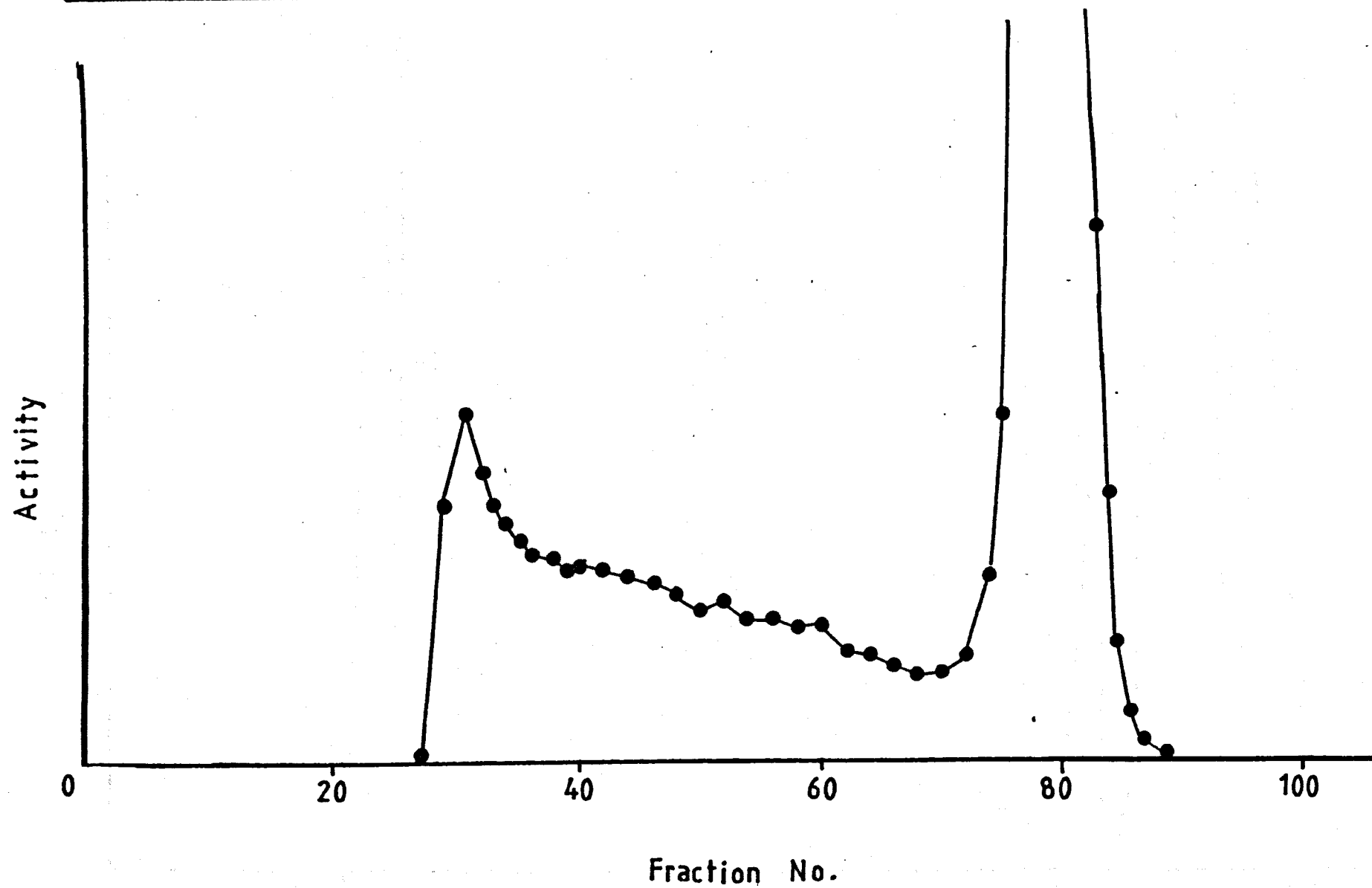


FIG. 9.12. SEPHADEX G-10 SEPARATION OF S1 REACTION MIXTURE

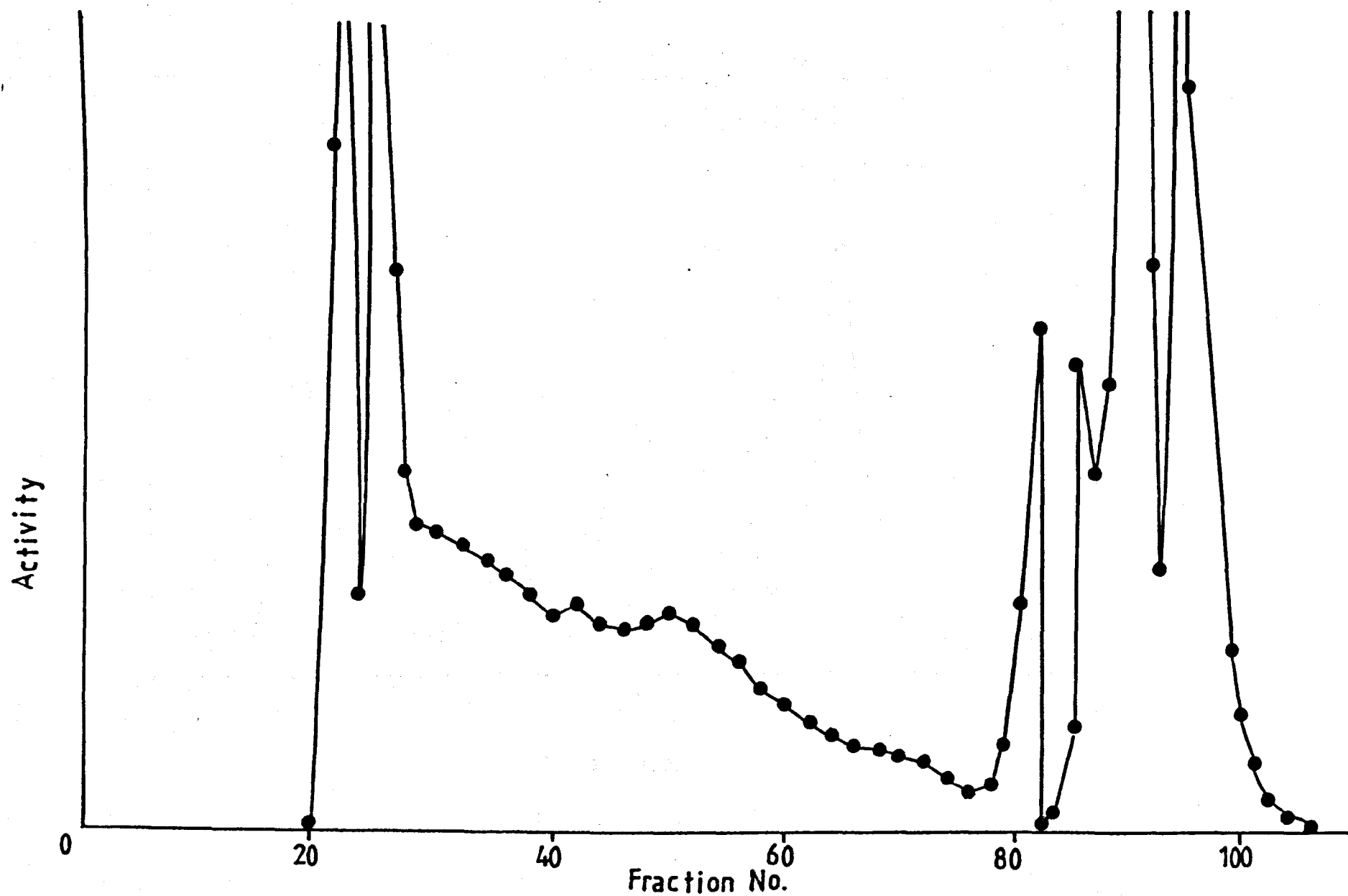


FIG. 9.13. ELECTROPHORESIS OF S1 [Fraction 35] AFTER G-25 SEPARATION

(See Fig. 9.11.)

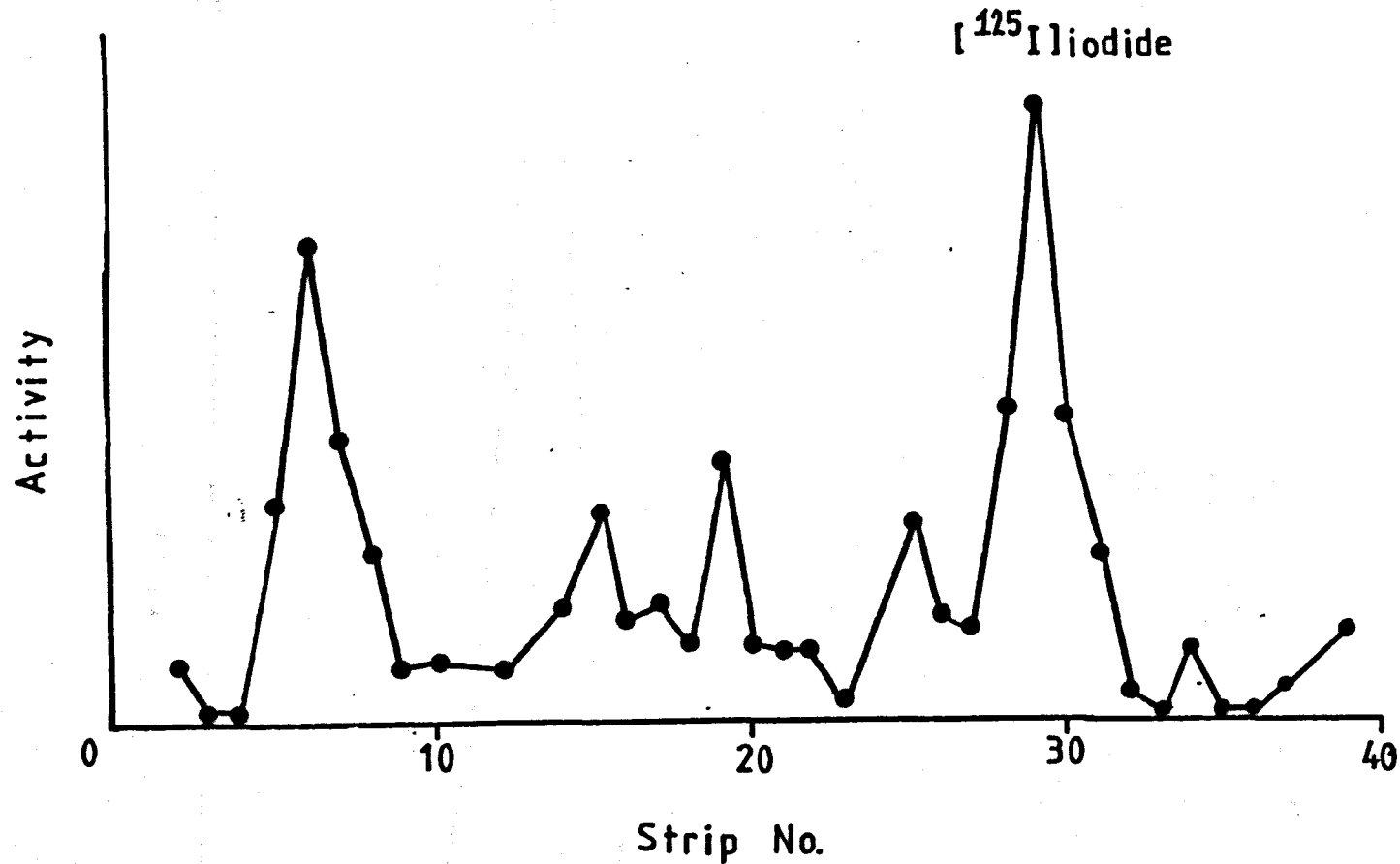


FIG. 9.14. ELECTROPHORESIS OF S1 [Fraction 22] AFTER G-10 SEPARATION

(See Fig.9.12.)

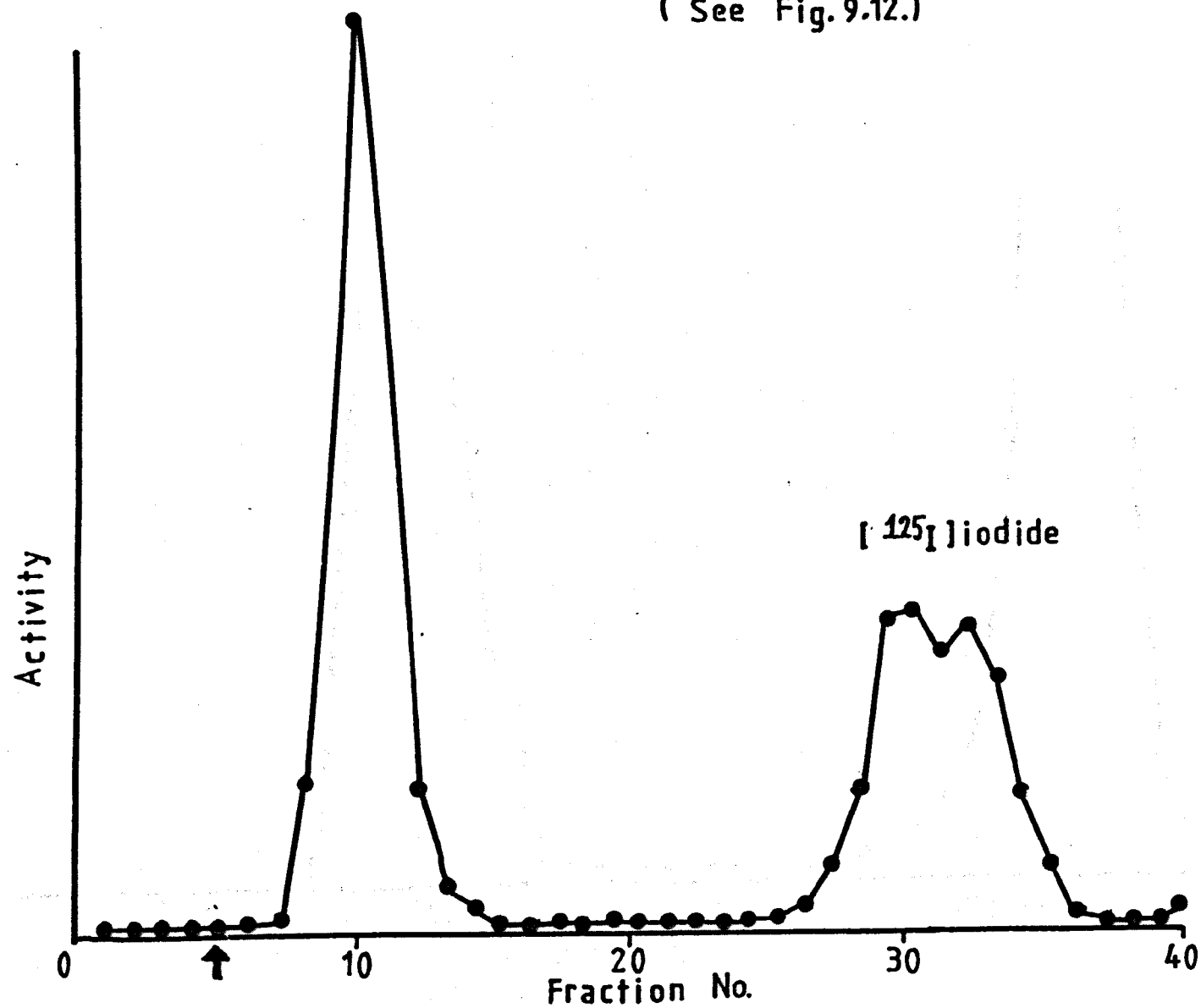


FIG. 9.15, SEPHADEX G-25 SEPARATION OF S3 REACTION MIXTURE

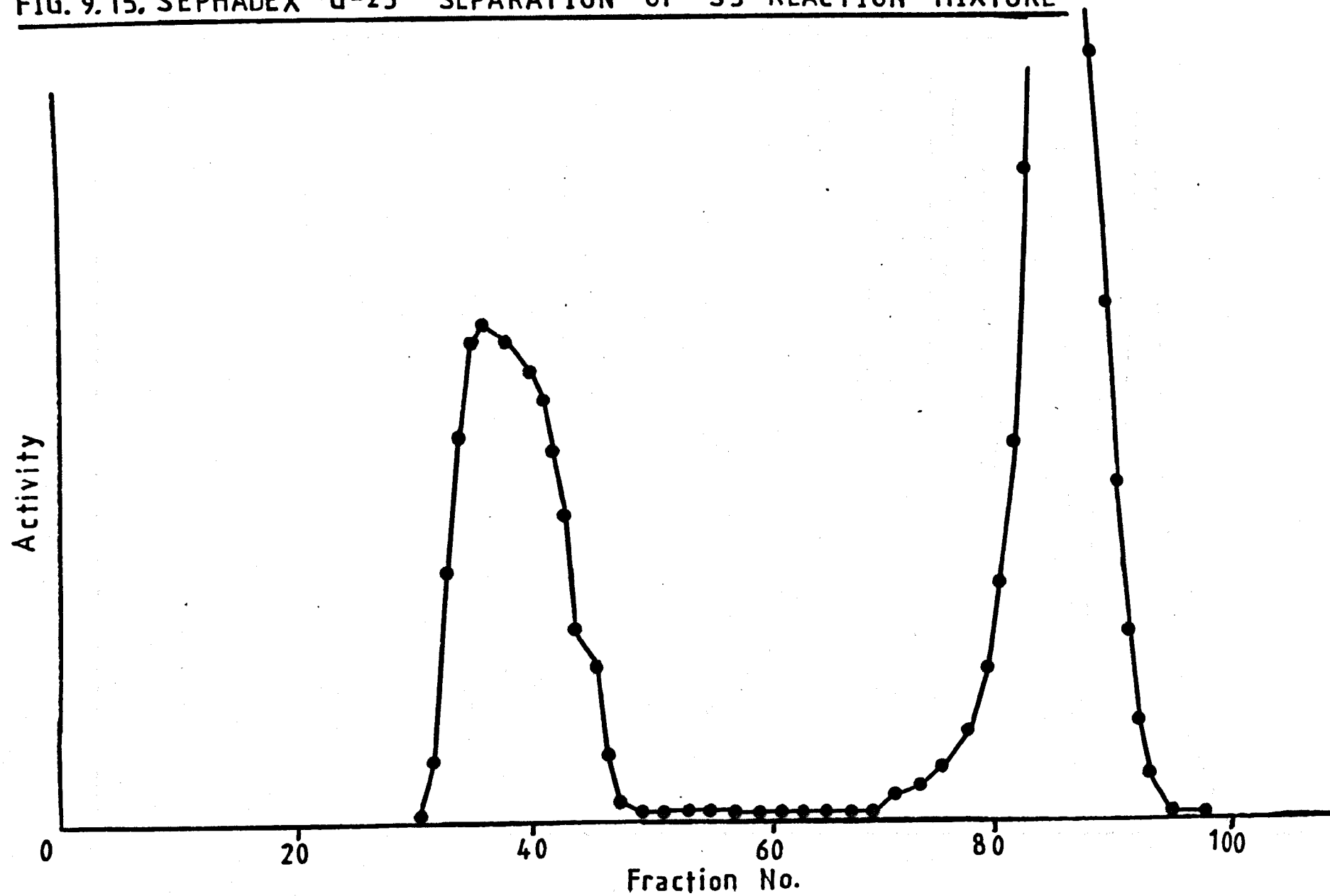


FIG. 9.16. SEPHADEX G-25 SEPARATION OF S4 REACTION MIXTURE

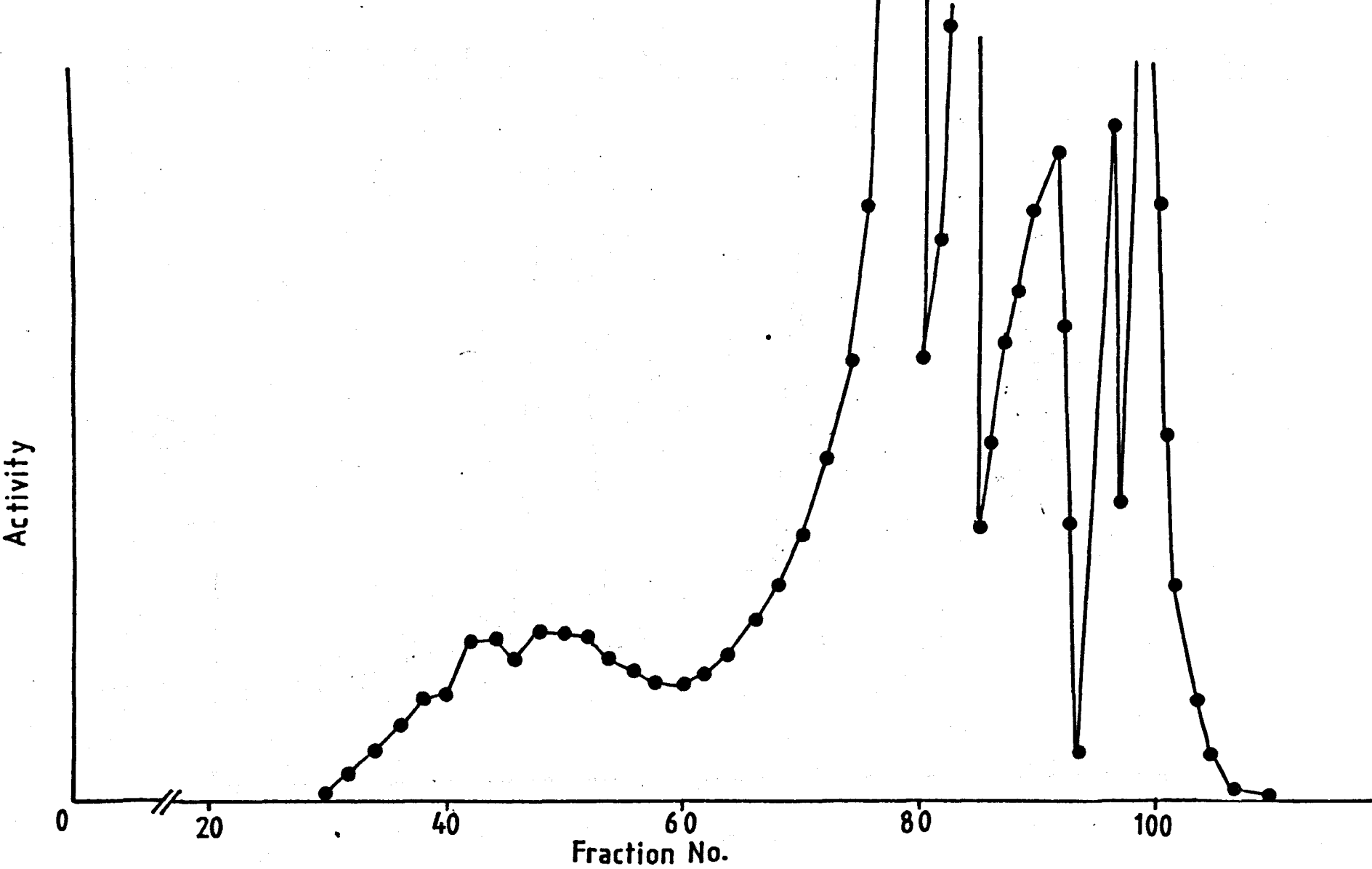


FIG.9.17. ELECTROPHORESIS OF S3 [Fraction 36] AFTER G-25 SEPARATION

(See Fig. 9.15.)

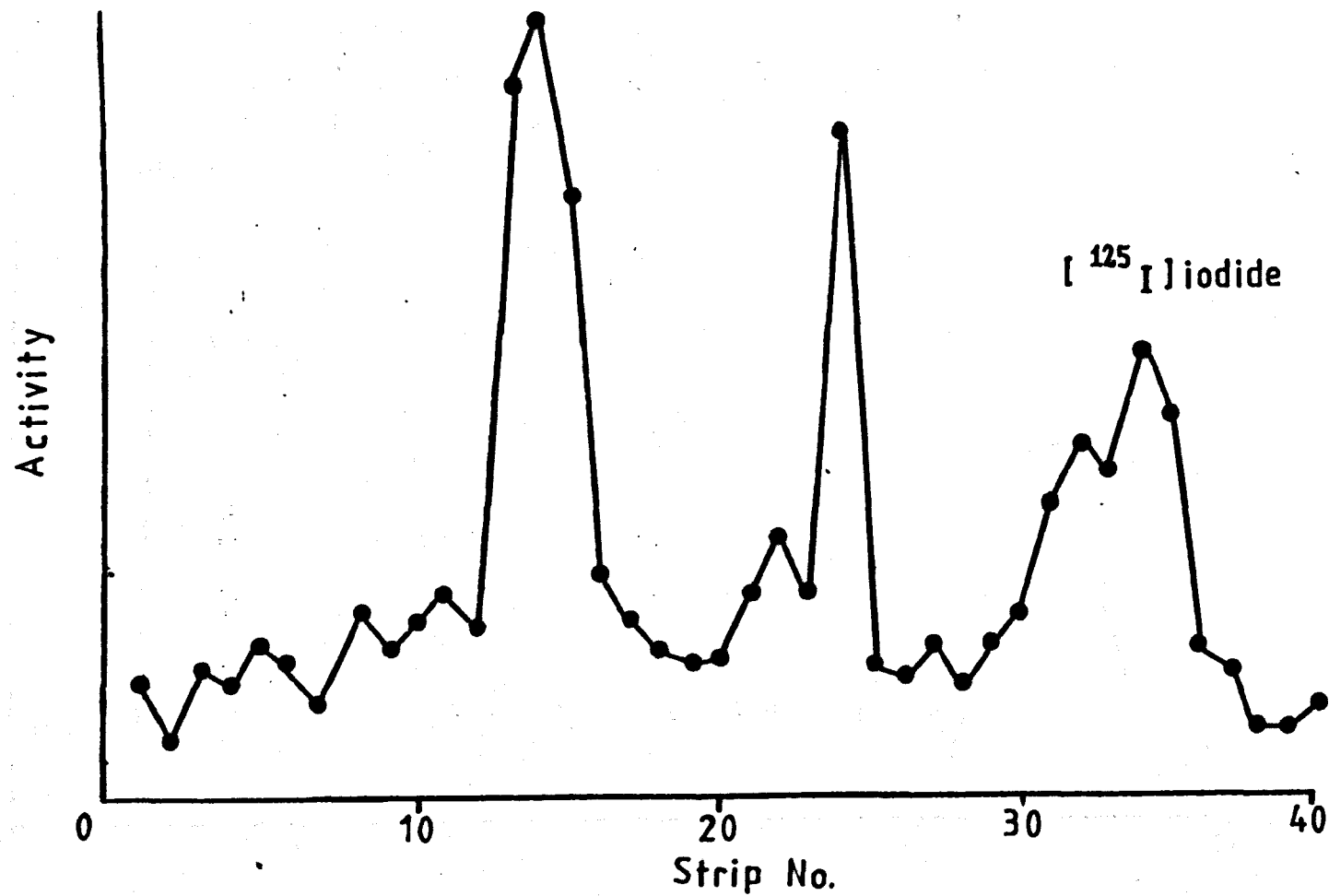


FIG. 9.18. ELECTROPHORESIS OF S4[Fraction 43] AFTER G-25 SEPARATION

(See Fig. 9.16.)

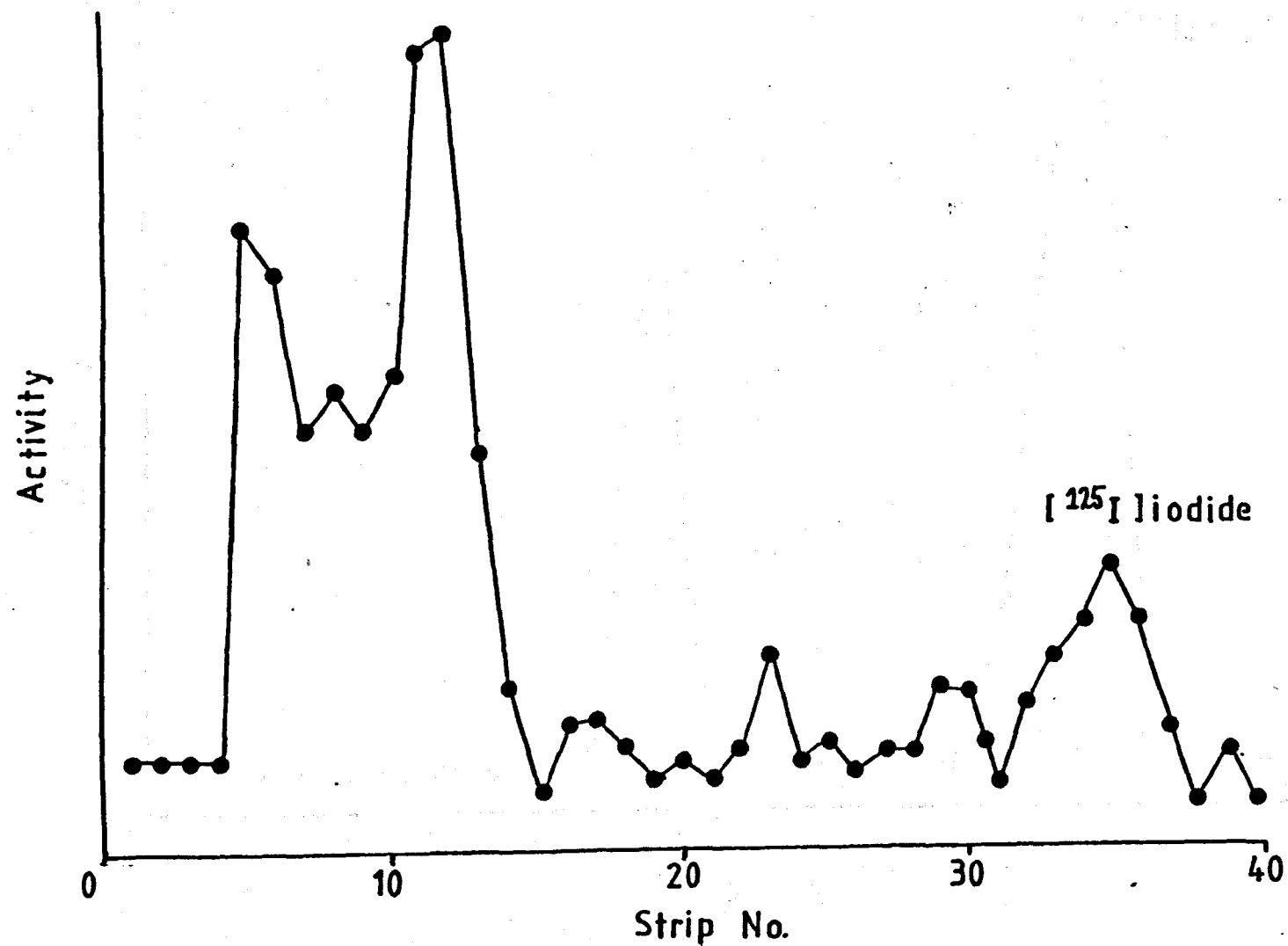


FIG. 9.19. ELECTROPHORESIS OF S4 [Fraction 75] AFTER G-25 SEPARATION

(See Fig. 9.16)

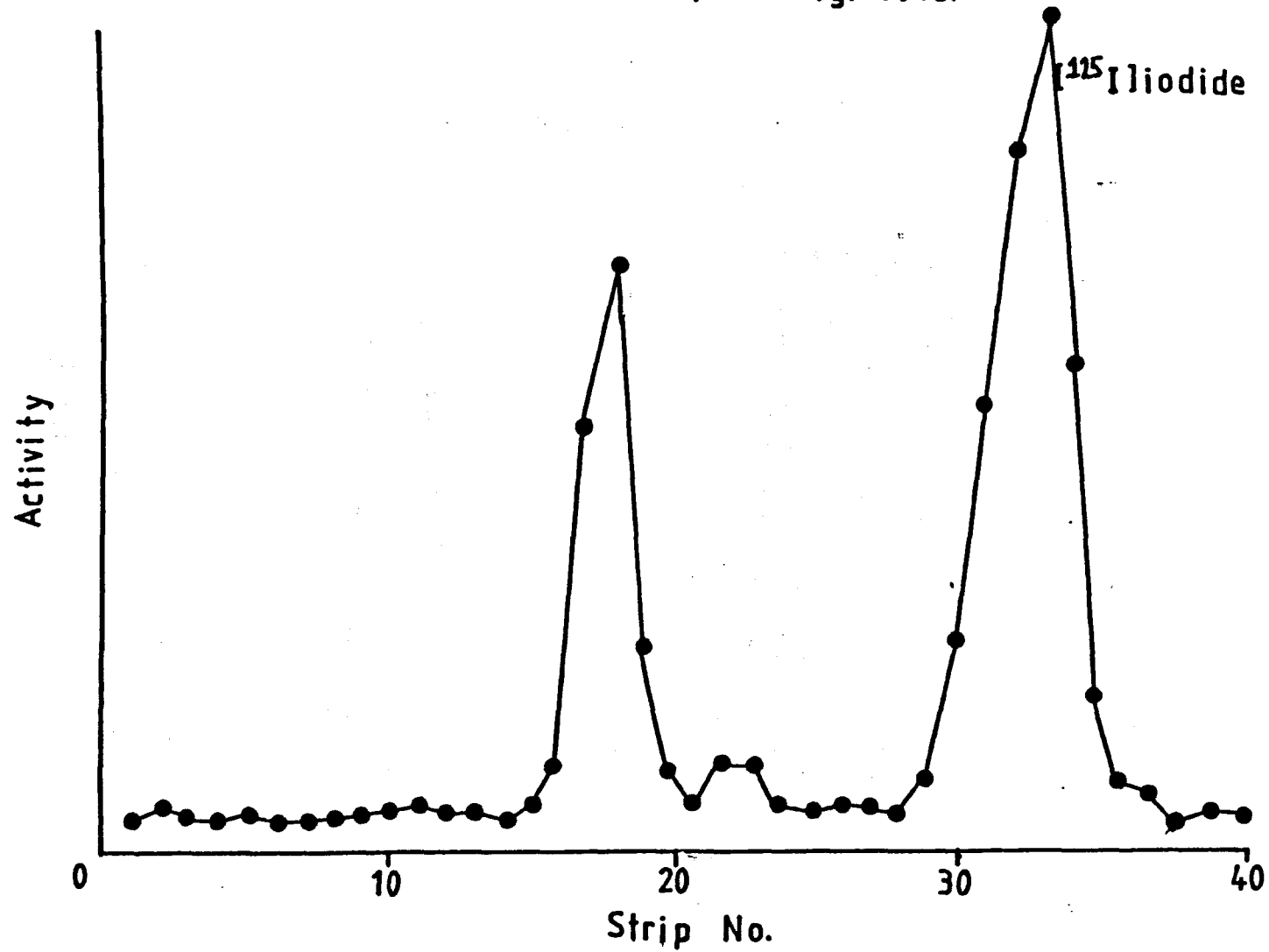


FIG. 9.20. ELECTROPHORESIS OF S1 FOLLOWING DIALYSIS

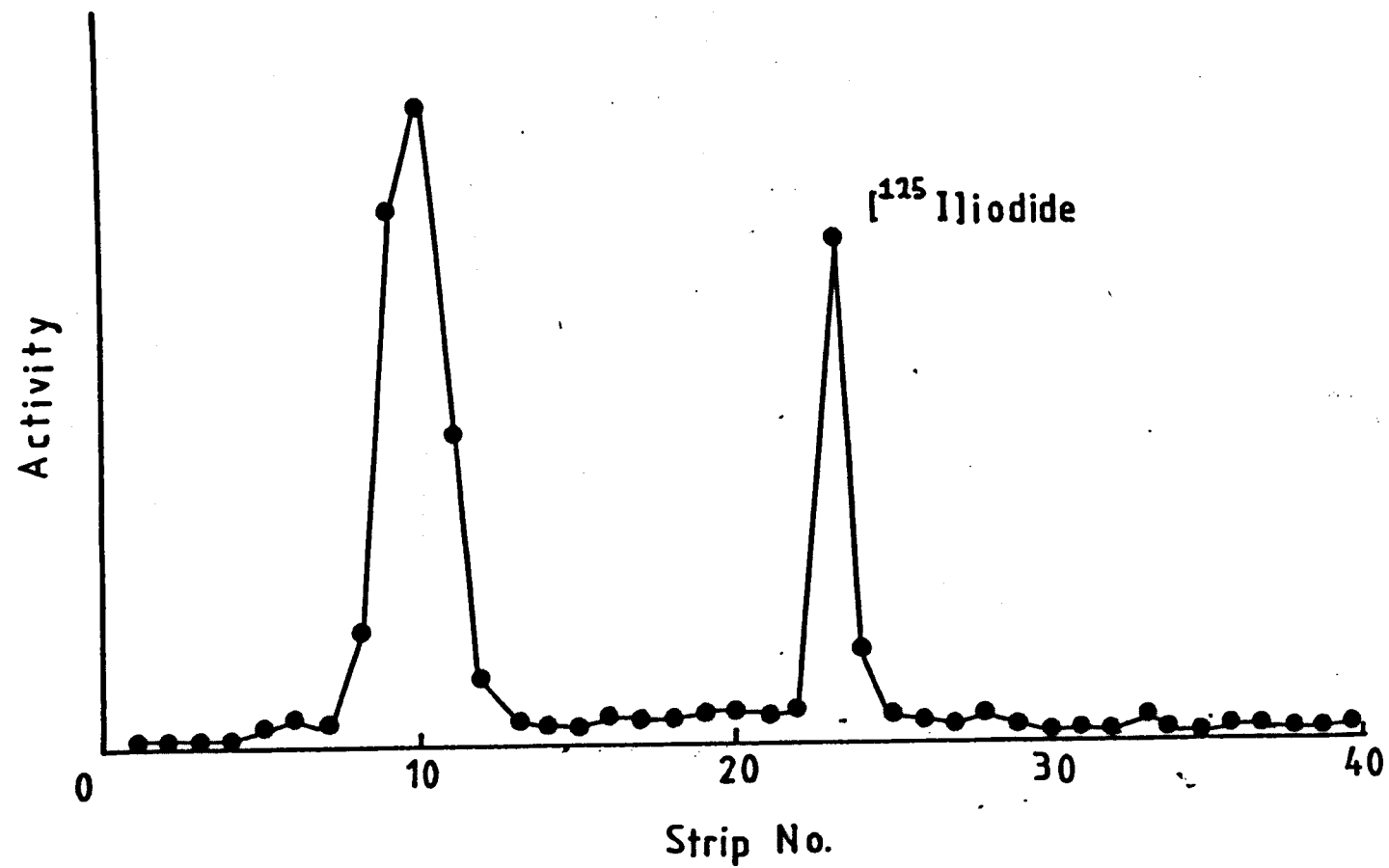


FIG. 9.21. ELECTROPHORESIS OF S2 FOLLOWING DIALYSIS

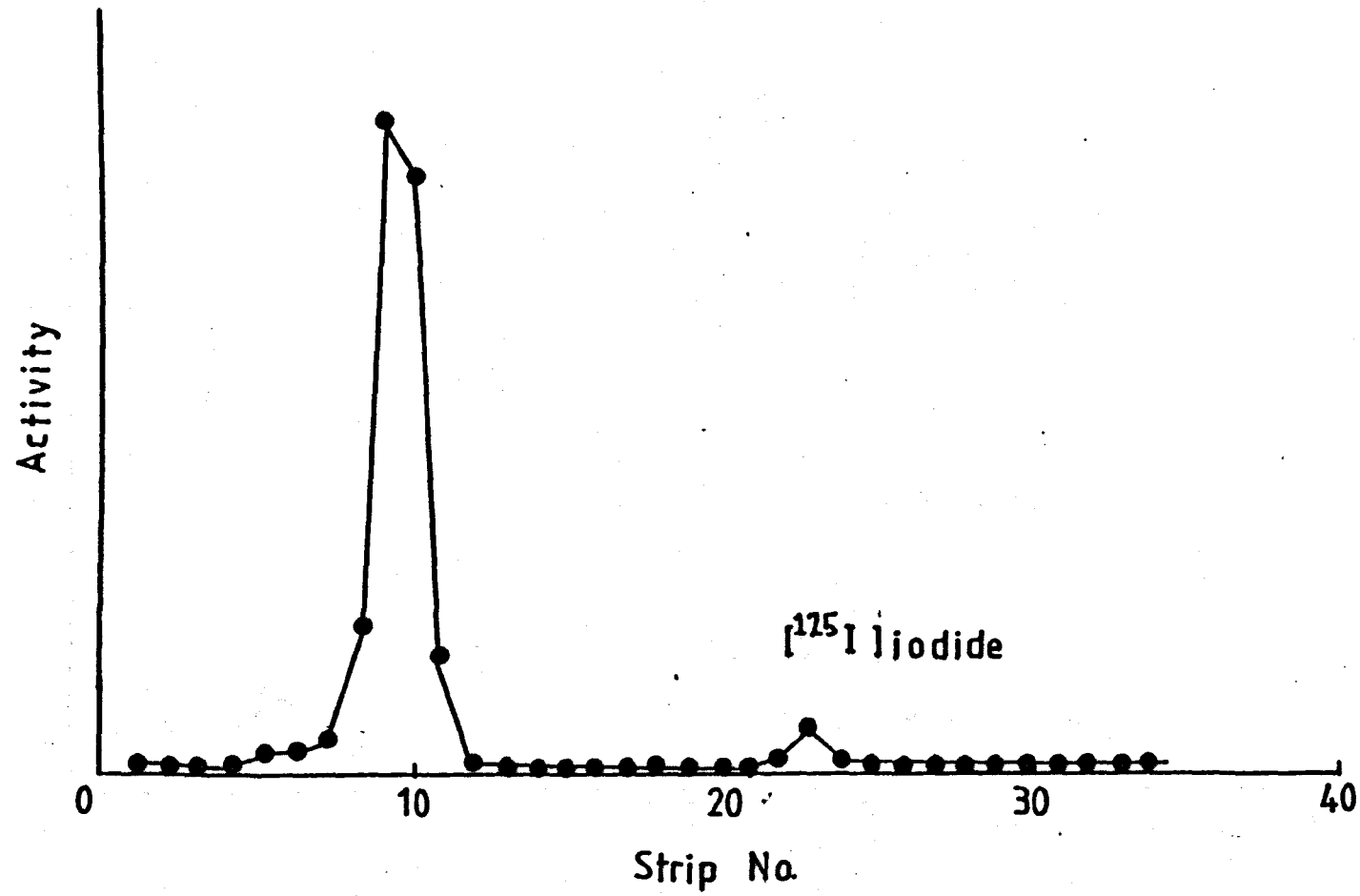


FIG. 9.22. ELECTROPHORESIS OF S3 FOLLOWING DIALYSIS

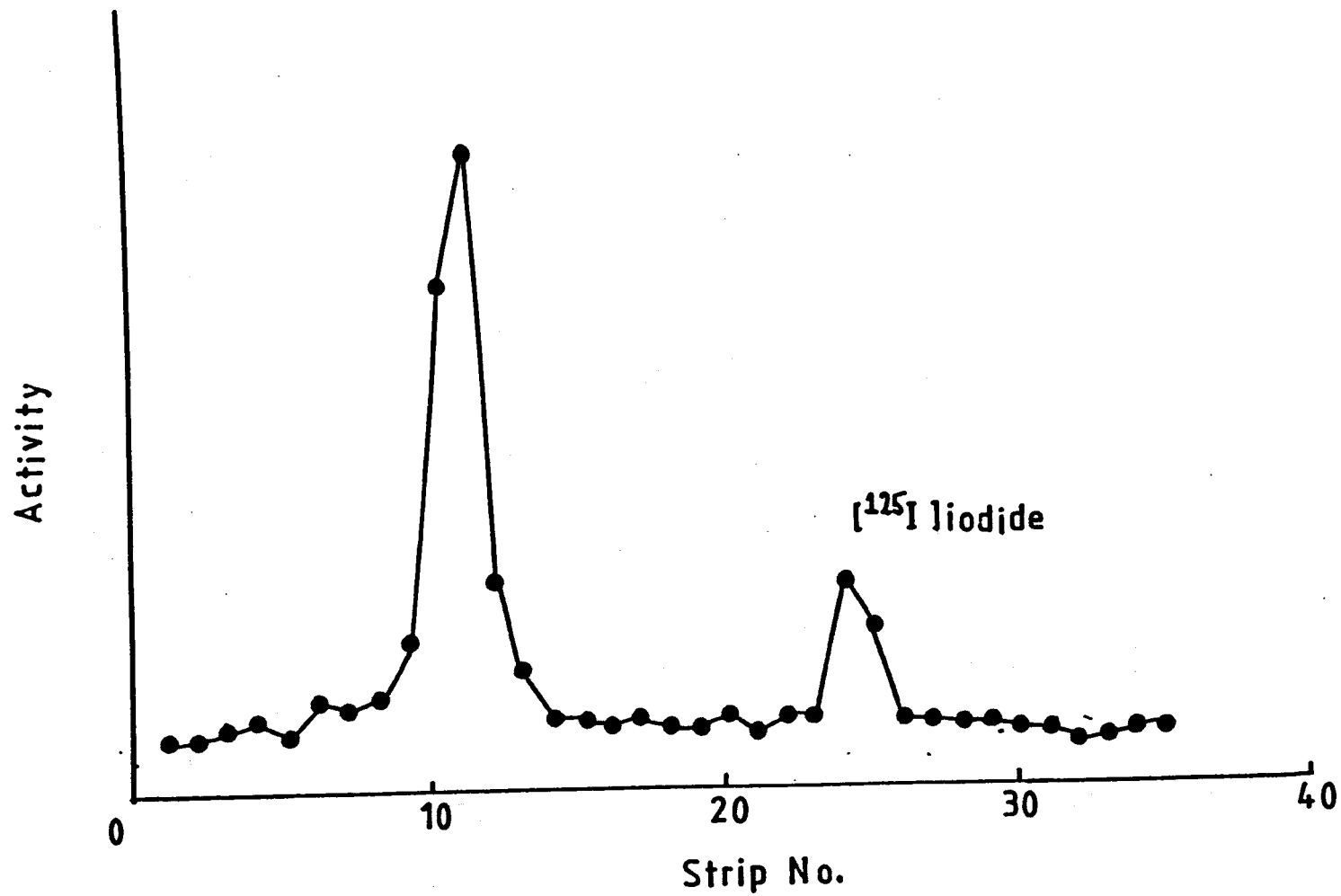


FIG. 9. 23. ELECTROPHORESIS OF S4 FOLLOWING DIALYSIS

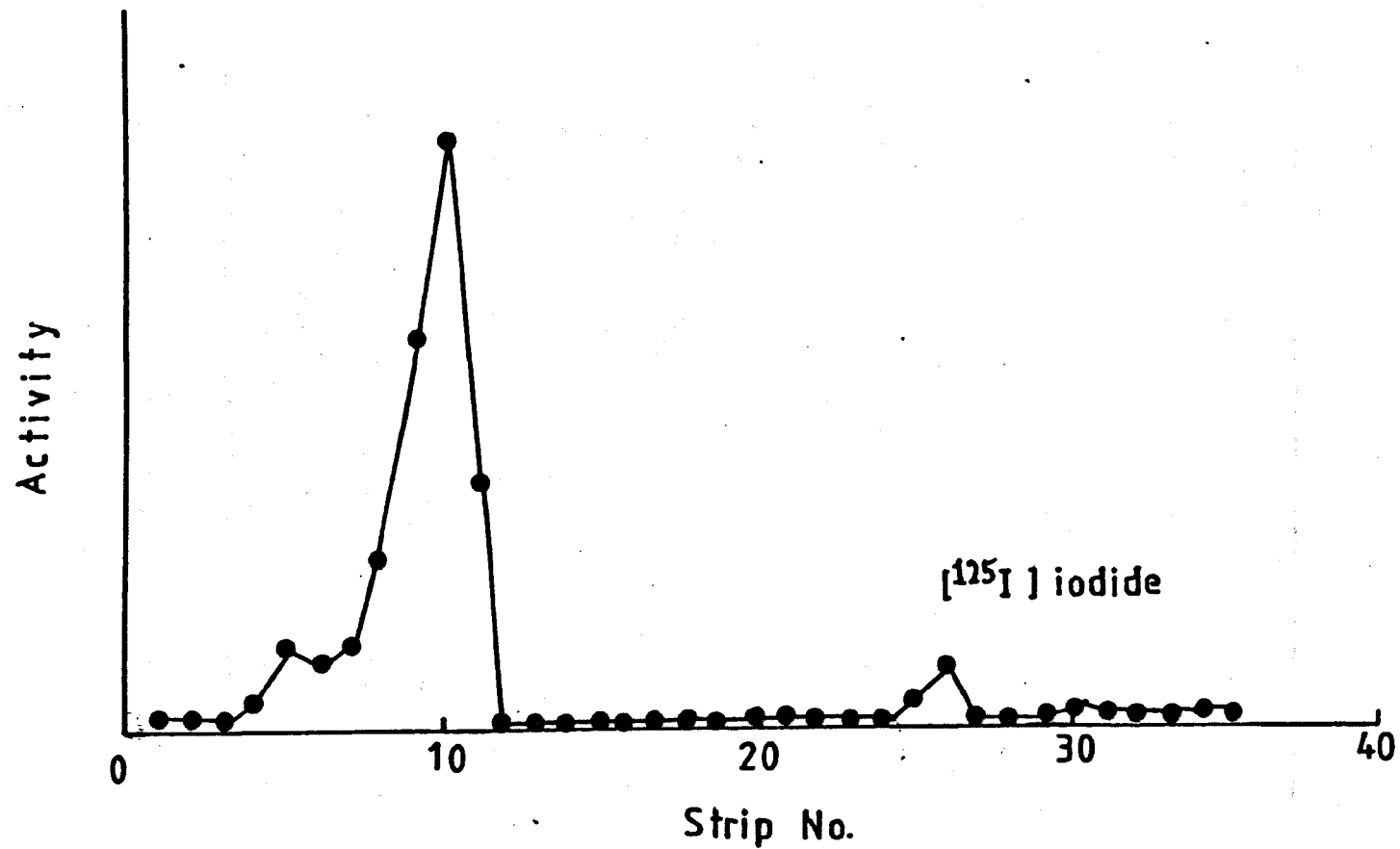


TABLE 9.2. EFFECT OF DIALYSIS AND SUBSEQUENT STORAGE ON THE
PERCENTAGE ^{125}I IODIDE IN S1 TO S4.

| | S1 | S2 | S3 | S4 |
|--------------------------------|-------|--------|--------|-------|
| Initially | 78.6% | 54.3% | 66.6% | 74.4% |
| Immediately Post Dialysis | 27% | 2.9% | 13.5% | 5.9% |
| <u>Time after Dialysis</u> | | | | |
| 1 Day | - | 3.0% | - | - |
| 11 Days | 35.2% | - | 14.8% | 6.5% |
| 12 Days | - | 7% | - | - |
| 16 Days | - | 9.2% | - | - |
| 19 Days | 43.0% | - | 20.0% | 10.3% |
| 26 Days | 28.7% | - | 32.31% | 7.39% |
| 27 Days | - | 14.0% | - | - |
| 35 Days | - | 16.27% | - | - |
| 42 Days | - | 15.05% | - | - |

Figures represent the percentage ^{125}I iodide in each ^{125}I -PVP preparation.

FIG. 9.24. DEIODINATION OF S1-S4 DURING STORAGE AT 4°C

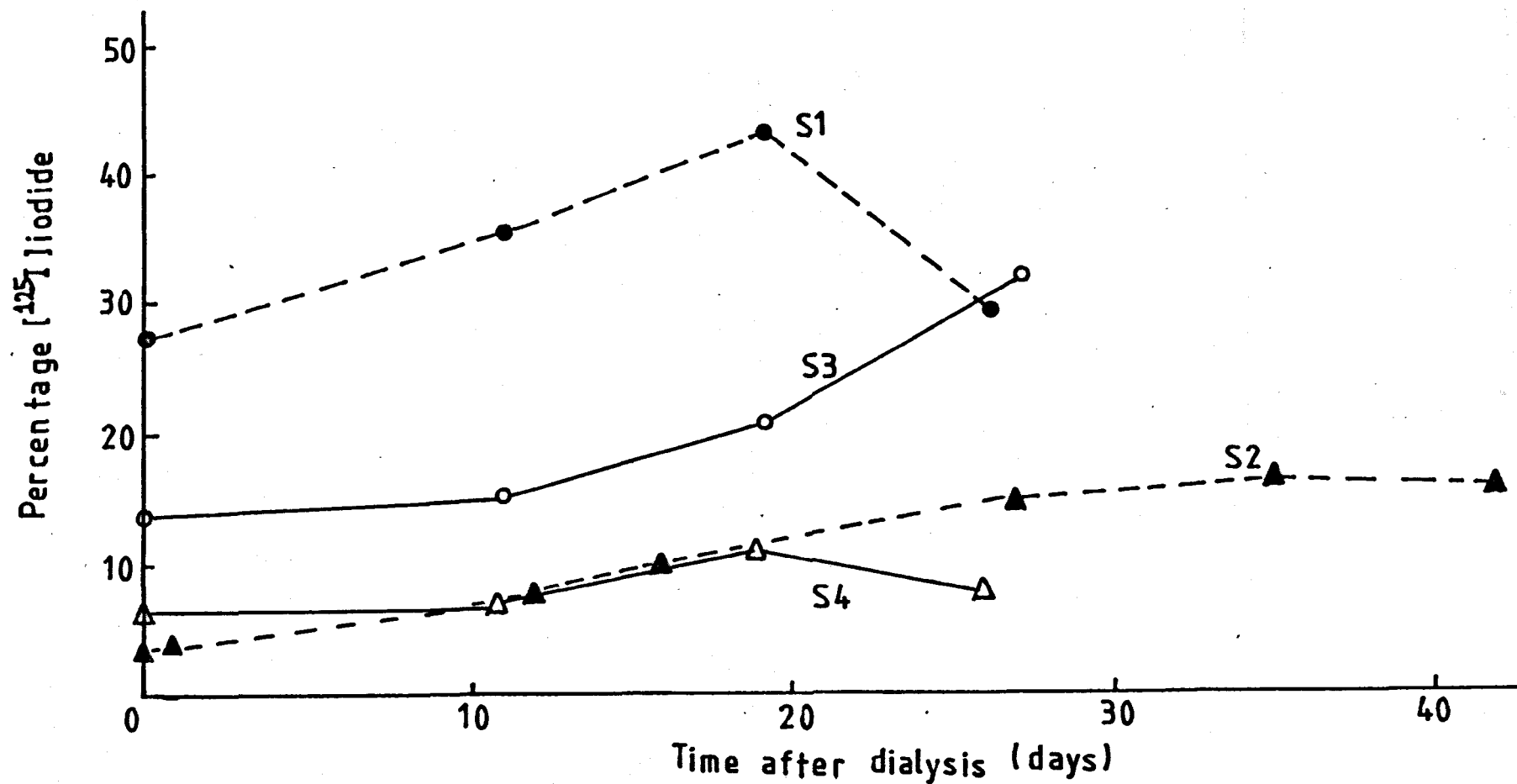


TABLE 9.3. EFFECT OF 6h INCUBATION AT 37°C ON ^{125}I IODIDE CONTENT
OF S1 TO S4.

| <u>PVP</u> | <u>Initially</u> | <u>After Incubation</u> |
|------------|------------------|-------------------------|
| S1 | 43.0% | 39.4% |
| S2 | 16.27% | 18.06% |
| S3 | 20.0% | 26.9% |
| S4 | 10.3% | 13.4% |

FIG. 9. 25. TISSUE-ASSOCIATION OF [125 I] IODIDE
MEASURED IN 3 SEPARATE EXPERIMENTS

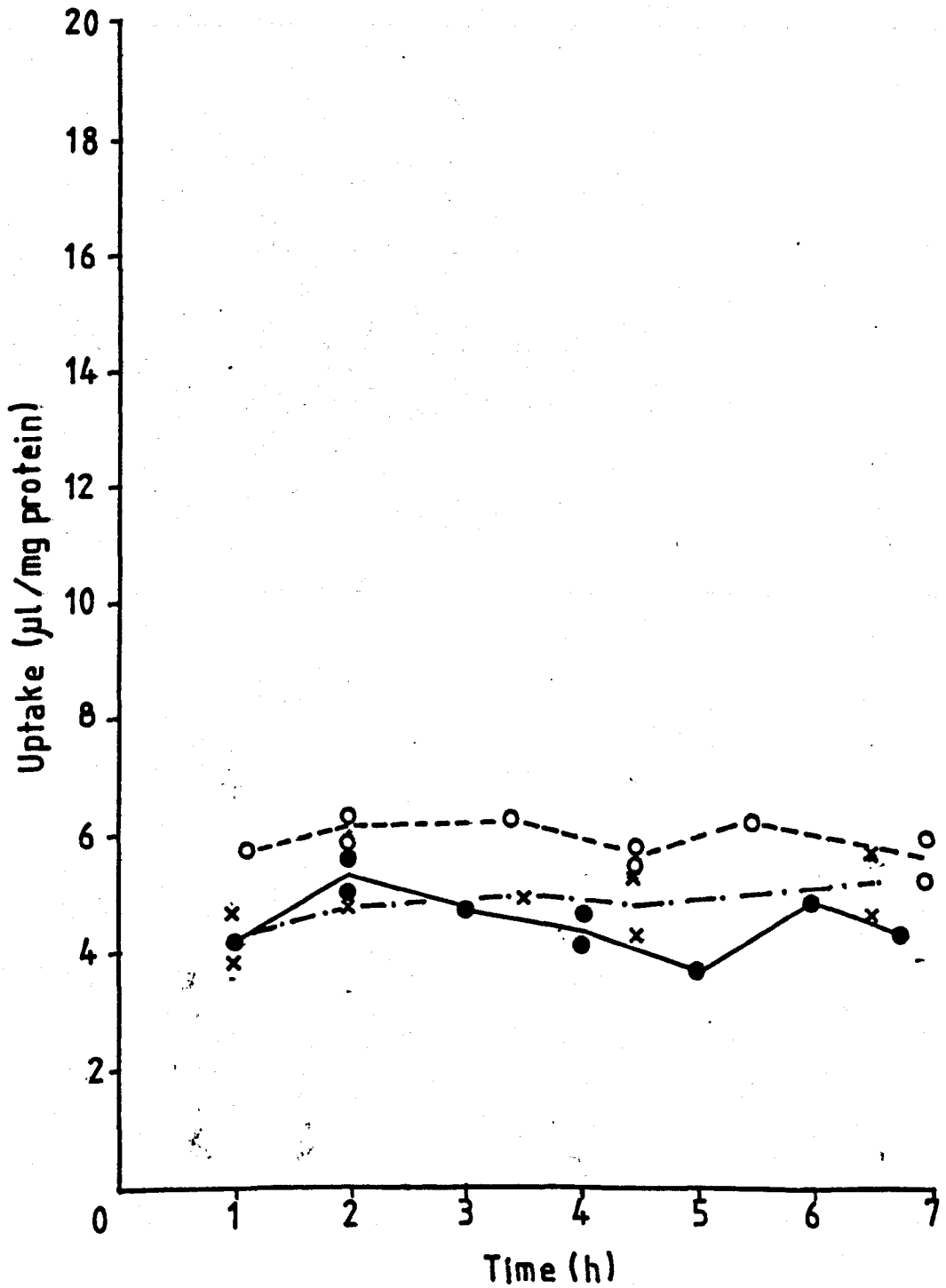


TABLE 9.4. THE UPTAKE OF ^{125}I -PVP IN THE PRESENCE OF LARGE
QUANTITIES OF ^{125}I IODIDE

| Experiment | Endocytic Index of ^{125}I -PVP ($\mu\text{l}/\text{mg}/\text{protein}/\text{h}$) | Endocytic Index of ^{125}I -PVP taken up in the presence of ^{125}I iodide ($\mu\text{l}/\text{mg}/\text{protein}/\text{h}$) | Percentage ^{125}I iodide in mixture |
|------------|--|---|---|
| 1 | - | 3.87 | 46% |
| 2 | 2.58 | 3.49 | 40% |
| 3 | 3.12 | 3.20 | 27% |
| 4 | 2.22 | 2.23 | 13% |
| MEAN | 2.64 ± 0.26 | 3.19 ± 0.35 | |

9.4. DISCUSSION

The method of Regoeczi (1976) was found to be a suitable technique for the radiolabelling of PVP samples S1 to S4, the labelling efficiency varying between 20% and 45%. Problems were encountered when various methods were used to try and isolate the ^{125}I -PVP from the remaining free ^{125}I iodide within the crude reaction mixtures. Although Regoeczi found anion-exchange chromatography quite adequate for this purpose, it is obvious from Figs. 9.9. and 9.10. that passage of crude samples of radioiodinated S1 and S3 through such a system did certainly not eliminate all the ^{125}I iodide, although the contamination was somewhat reduced. A Sephadex system offered the obvious alternative to this method and certain preparations were run down either Sephadex G-25, Sephadex G-10 or both. Samples of crude reaction mixture of S1 separated quite well, but there was a large component of low molecular weight material (Figs. 9.11. and 9.12.). However, subsequent electrophoresis of the supposedly ^{125}I -PVP fraction revealed a significant proportion of free ^{125}I iodide. This observation is somewhat puzzling unless the ^{125}I -PVP decomposes rapidly after elution from Sephadex.

S3 separated into two distinct peaks on Sephadex G-25, but once again electrophoresis of a supposedly pure polymer fraction revealed considerable ^{125}I iodide contamination. In contrast the Sephadex G-25 elution of S4 produced a very strange pattern and this may have been due to its size distribution (mean molecular weight 700,000). The PVP used to saturate the gel before a run (to stop non-specific binding of PVP) was only of mean molecular weight 40,000, so it is quite likely that S4 might interact with the gel, and this would result in distortion of the elution pattern. Electrophoresis of two fractions from this Sephadex elution of S4 showed an increase in the percentage of ^{125}I iodide, and also an interesting shift in the mobility of S4, with higher fraction number i.e. when approaching the iodide peak.

It is very difficult to explain the differing electrophoretic mobilities of the components within the ^{125}I -PVP preparations. This is particularly noticeable in the crude reaction mixtures of S2 and S4 (Figs. 9.5. and 9.7.). Preparation S2 shows two distinct peaks moving towards the cathode which are both presumably ^{125}I -PVP and certainly migrate more slowly than $[\text{}^{125}\text{I}]$ iodide. S4 displays similar characteristics and three ^{125}I -PVP peaks are clearly visible. Perhaps the different ^{125}I -PVP species represent molecules that have undergone differing degrees of iodination. It is interesting to note that following dialysis this heterogeneity disappears and the familiar unimodal distribution of ^{125}I -PVP, with little electrophoretic mobility, returns for all the PVP size distributions (Figs. 9.20. to 9.23.).

Dialysis may seem to be the obvious choice for the removal of free $[\text{}^{125}\text{I}]$ iodide from the ^{125}I -PVP preparations, but initially this method was avoided as it was hoped that a standard preparative method could be used for all the compounds under investigation, and it was considered doubtful that the lower molecular weight fraction of S1 would remain confined within the dialysis membrane. This problem however did not materialize. Following dialysis, analysis of the PVP preparations showed that the percentage free $[\text{}^{125}\text{I}]$ iodide was considerably reduced, being 27%, 3%, 14% and 6% in the samples S1 to S4 respectively. The relatively large quantity of $[\text{}^{125}\text{I}]$ iodide remaining in preparation S1 was worrying, but the levels in the other preparations were deemed acceptable.

During storage at 4°C there was some spontaneous deiodination of all the ^{125}I -PVPs (Table 9.2.), the maximum loss being 0.58%/day in the case of S3. These observations contrast with the report of Regoeczi (1976) where it was stated that the ^{125}I -PVP prepared (mean molecular weight 33,000) remained completely stable over 4 weeks when stored at 5°C and only released 5% of the bound $[\text{}^{125}\text{I}]$ iodide when kept at 37°C .

for this length of time. Although the deiodination of the ^{125}I -PVPs prepared here was certainly undesirable, the rate of loss of bound $[\text{}^{125}\text{I}]$ iodide was sufficiently slow to make the use of the preparations possible if some method could be devised to compensate for the radioactivity in the preparation that was directly related to $[\text{}^{125}\text{I}]$ iodide and not ^{125}I -PVP.

Visceral yolk sacs incubated in the presence of $[\text{}^{125}\text{I}]$ iodide showed a constant level of tissue-associated radioactivity over the 6.5h culture period (Fig.9.25.). It seems very likely that iodide would be small enough to diffuse through the membranes into the yolk sac cells, and these results are quite consistent with this interpretation. The quantities of iodide (in terms of $\mu\text{l}/\text{mg}$ protein) which appeared to be tissue-associated were considerably lower than one would expect if an equilibrium was reached between tissue and culture medium radioactivities, but this is not suprising as the yolk sacs are thoroughly washed (3 x 2min) during the sampling procedure, during which time the iodide has ample opportunity to diffuse back out of the tissue into the washing solution. Having established that $[\text{}^{125}\text{I}]$ iodide is not itself progressively accumulated by the yolk sac, it seemed likely that the pinocytic capture of ^{125}I -PVP could be adequately monitored in the presence of considerable quantities of $[\text{}^{125}\text{I}]$ iodide, as long as the percentage iodide in the preparation was a known quantity. This is obviously not the ideal situation for the quantification of uptake of ^{125}I -PVP, but it is workable if it proves impossible to produce "clean", stable ^{125}I -PVP preparations within the laboratory.

The uptake of Amersham ^{125}I -PVP was investigated in the the presence of known quantities of $[\text{}^{125}\text{I}]$ iodide and it was found that the Endocytic Index of ^{125}I -PVP measured under such conditions was marginally higher than that of the same ^{125}I -PVP without addition (Table 9.4.). The mean Endocytic Indices were 3.19 ± 0.35 and $2.64 \pm 0.26 \mu\text{l}/\text{mg}$ protein/h respectively and, although these differences are probably not significant,

it is interesting to note the apparent correlation between increasing concentration of iodide and increasing Endocytic Index. (Confirmation of this trend would necessitate the replication of these experiments with precisely controlled levels of $[^{125}\text{I}]$ iodide in the culture medium). As would be expected the uptake of ^{125}I -PVP with time retains linearity in the presence of $[^{125}\text{I}]$ iodide, but it should be noted that no correction was made for the tissue radioactivity that represented $[^{125}\text{I}]$ iodide. This means that the measured amount of ^{125}I -PVP at each time point ($\mu\text{l}/\text{mg}$ protein) will be an over estimate, but as the tissue-association of $[^{125}\text{I}]$ iodide is non-progressive the uptake value for each yolk sac will be affected equally and therefore the rate of accumulation of ^{125}I -PVP ($\mu\text{l}/\text{mg}$ protein/h) will not be biased in any way. The system becomes invalid if the substrate is not stable during culture, but the radiolabelled preparations of S1 to S4 were shown to be acceptably stable during a 6h incubation in medium 199 containing 10% calf serum at 37°C (Table 9.3.). Although far from ideal substrates, the ^{125}I -PVP preparations made in the laboratory were usable as such and the rate of pinocytosis of S1 to S4 is reported in the following Chapter.

CHAPTER 10

UPTAKE OF ^{125}I -PVPS OF DIFFERENT MOLECULAR

SIZE DISTRIBUTION BY THE RAT VISCERAL YOLK SAC.

10.1. INTRODUCTION

The relationship between substrate size and the rate of pinocytosis of a substrate has never been effectively investigated. Bartoleyns & Baudhuin (1976) and Kooistra et al., (1977) examined the endocytosis of ribonuclease A monomer and oligomers and both groups found that there was a correlation between increasing size and endocytic uptake. However, these data are not particularly relevant to the question of size and pinocytic uptake as the first study used two relatively small ribonuclease preparations (monomer and dimer) and the second, although employing a larger range of substrate sizes, was carried out in vivo so that the substrate was exposed to cells with various endocytic capacities.

Ideally to study the relationship between substrate size and pinocytic uptake, a fluid-phase marker with characterized and preferably completely distinct size distributions is required. Here four different ^{125}I -PVP samples were used for this purpose and the molecular weight distributions of these polymers has already been given (see Section 9.2.3.). The method of radiolabelling these polymers is described in the preceding Chapter.



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10.2. RESULTS

The quantitation of uptake of the ^{125}I -PVPs, S1 to S4, was carried out using basically the same method as that described in Section 2.3. . Immediately before using S1 to S4 in the culture system a small sample of each preparation was subjected to electrophoresis (see Section 2.8.) and the percentage $[\text{}^{125}\text{I}]$ iodide in the preparation determined. Before calculation of the rate of uptake of S1 to S4 the medium radioactivity measured in each culture flask was corrected by taking from this value the percentage that was attributable to $[\text{}^{125}\text{I}]$ iodide. No allowance was made for tissue-association of $[\text{}^{125}\text{I}]$ iodide, but the non-progressive nature of this accumulation means that the Endocytic Indices for ^{125}I -PVP are absolute values. (see Fig. 9.23. and Section 9.4.)

Fig. 10.1. shows the uptake of S1, S3 and S4 by yolk sacs incubated in separate flasks for a period of 6.5h. (Unlike the other experiments reported in this thesis uptake was measured at only one time interval and the results are expressed as a mean \pm S.E. of the uptake values obtained from three or four yolk sacs.) In this experiment the preparations of ^{125}I -PVP used correspond to those that were labelled and tested extensively in the previous Chapter (i.e. preparation 1, see Table 9.1.)

For more rigorous investigation of the uptake of S1 to S4, a second batch of radiolabelled polymers was made (preparation 2, see Table 9.1.) . The Endocytic Indices calculated for S2, S3 and S4 are shown in Table 10.1. . Unfortunately the specific activity of S1 was so low that the margin of error became very high and the correlation coefficients observed for these experiments were unacceptably low. However, when the individual uptake points were averaged out at each time interval, the data did show a trend. Fig. 10.2. shows the mean uptake plots obtained for all four ^{125}I -PVPs.

The effect of unlabelled samples of each size distribution of PVP (100 $\mu\text{g/ml}$) on the uptake of Amersham ^{125}I -PVP was investigated in a single experiment and the results are shown in Table 10.2.. In the presence of any

FIG. 10. 1. UPTAKE OF S1, S3 & S4 (preparation 1)
AFTER 6.5h OF INCUBATION

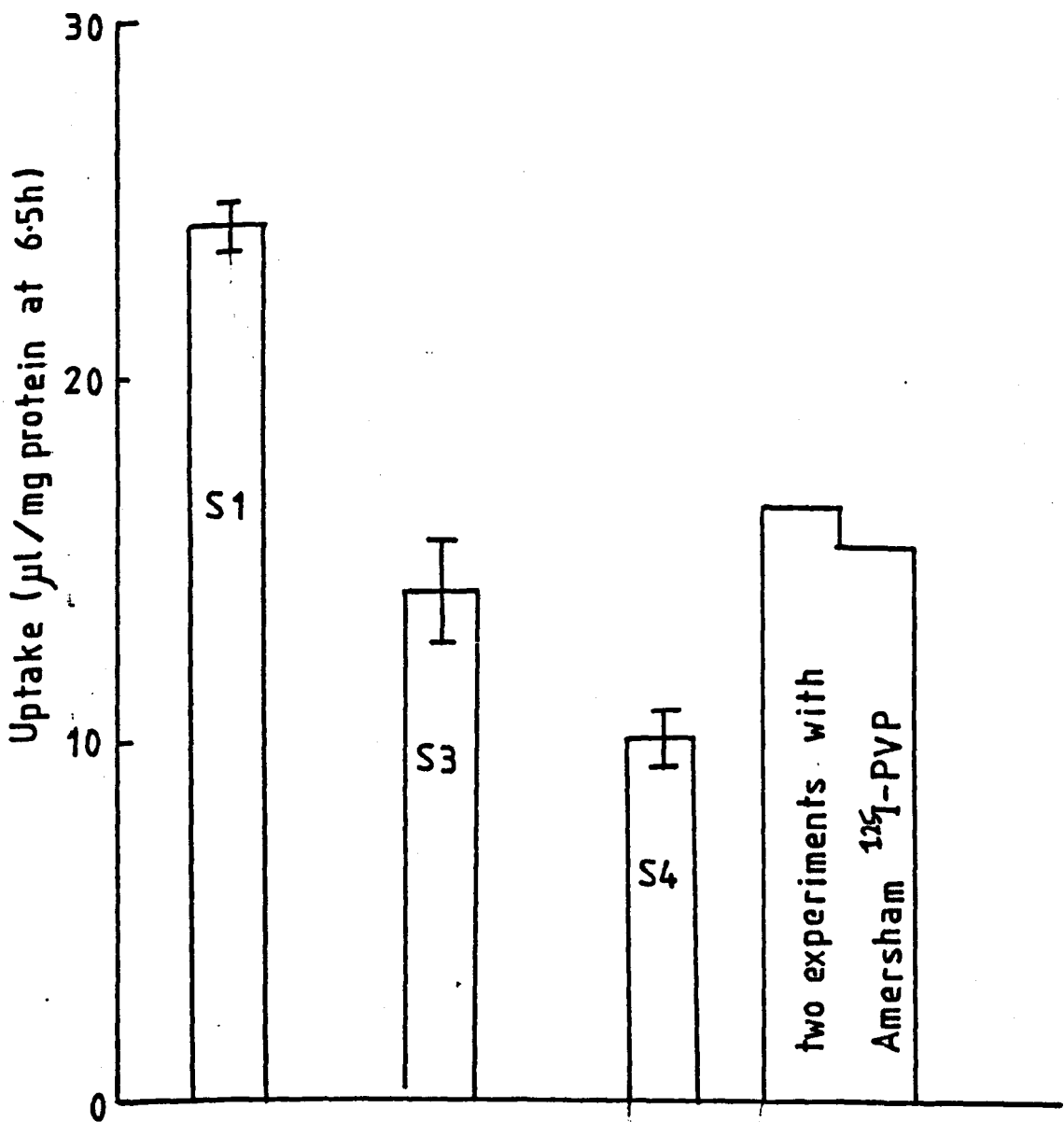


TABLE 10.1. RATE OF UPTAKE OF ^{125}I -PVPs of KNOWN SIZE DISTRIBUTION.

| PVP | Endocytic Index ($\mu\text{l}/\text{mg}$ protein/h) | Mean Endocytic Index (\pm SE) | Endocytic Index of Matched Control ($\mu\text{l}/\text{mg}$ protein/h) | Percentage Control | Mean Percentage of Matched Control |
|-----|---|-------------------------------------|--|-----------------------|---------------------------------------|
| S2 | 2.43 | 2.14 ± 0.14 | 2.76 | 88 | 104% |
| | 2.06 ⁺ | | 1.55 ⁺ | 133 ⁺ | |
| | 1.77 | | 1.77 | 100 | |
| | 2.29 | | 2.41 | 95 | |
| S3 | 1.62 ⁺ | 1.46 ± 0.18 | 1.55 ⁺ | 105 ⁺ | 68% |
| | 0.92 | | 1.77 | 52 | |
| | 1.90 | | 2.41 | 79 | |
| | 1.72 | | 2.71 | 63 | |
| | 1.16 | | 2.71 | 43 | |
| S4 | 1.45 | 1.13 ± 0.13 | 2.76 | 52 | 53% |
| | 0.89 | | 1.55 | 54 | |
| | 0.96 | | 1.77 | 54 | |
| | 1.23 | | 2.41 | 51 | |

+ These data were obtained using yolk sacs of either 16.5 or 18.5 days gestational age.

FIG. 10.2. UPTAKE OF THE DIFFERENT MOLECULAR
SIZED ^{125}I - PVPS, S1-S4 (preparation 2)

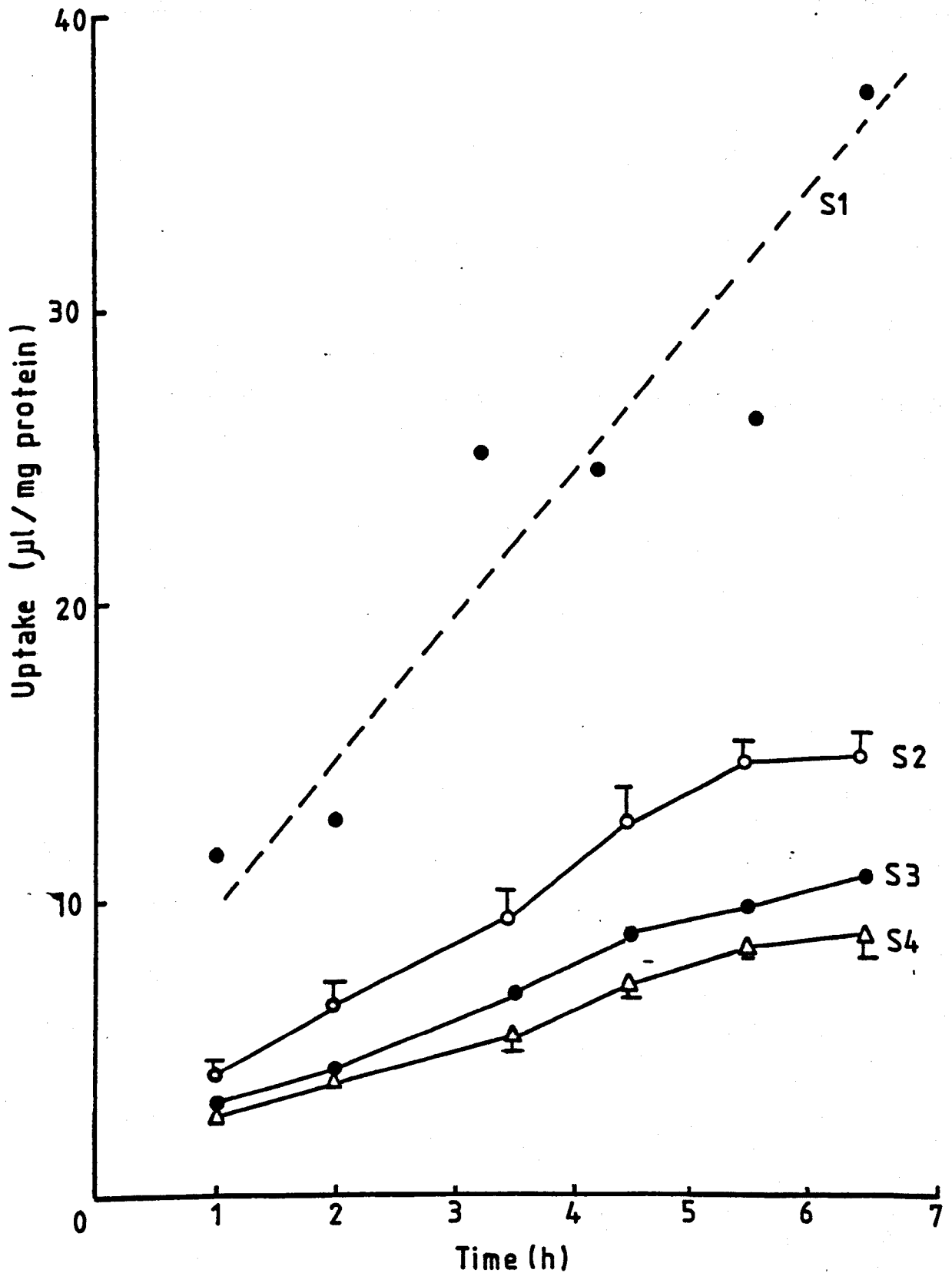


TABLE 10.2. EFFECT OF THE PVPs S1, S2, S3, AND S4 (100 μ g/ml)
ON THE UPTAKE OF AMERSHAM 125 I-PVP (2.8 μ g/ml)

| PVP No. (100 μ g/ml) | Endocytic Index of Amersham 125 I-PVP (μ l/mg protein/h) | Percentage of Endocytic Index estimated without addition |
|-----------------------------|--|--|
| - | 2.45 | - |
| S1 | 1.93 | 79 |
| S2 | 2.16 | 88 |
| S3 | 2.23 | 91 |
| S4 | 2.03 | 83 |

of the compounds S1 to S4 the Endocytic Index of Amersham ^{125}I -PVP was slightly lower than that measured in their absence. However, the Endocytic Indices obtained for Amersham ^{125}I -PVP all fell well within the normal range (see Table 3.1.) even in the presence of unlabelled PVP.

10.3. DISCUSSION

The Endocytic Index of the ^{125}I -PVP, S2 was not significantly different from that measured in matched control experiments using ^{125}I -PVP purchased from the Radiochemical Centre, Amersham (see Table 10.1.). Although the average molecular weights of these two preparations are not very different, being 50,000 and 33,000 respectively, the range of the molecular weight distributions are considerably different and this can be seen in Figs. 3.5. and 9.1. . Unlabelled S2 has a much larger size distribution, ranging from low molecular weight species of approximately 1,000 up to very high molecular weight species of 1000,000. Amersham ^{125}I -PVP is composed of polymers entirely within the molecular weight range 10,000 - 80,000. The observed similarity in the Endocytic Indices of these two ^{125}I -PVP preparations may indicate that there is not preferential labelling of S2 at the extremities of the molecular weight range. This would be consistent with the labelling efficiencies observed in Chapter 9 (see Table 9.1.), where it was noted that S2 had the optimum labelling efficiency.

The rate of pinocytosis of the ^{125}I -PVP, S3, was less than that measured for Amersham ^{125}I -PVP, the mean percentage of the matched control being 68%. There was some variability in the Endocytic Index measured for S3, but in four out of five experiments the rate of uptake was lower than that of the Amersham control. The average molecular weight of the unlabelled S3 preparation is 84,000, which is not appreciably higher than that of S2 but over twice that of Amersham ^{125}I -PVP. Unfortunately the size distribution of this preparation was not available.

Preparation S4 is composed of much larger polymers, the average molecular weight being 700,000. The rate of uptake of S4 was less than that obtained for S3 and the Endocytic Indices were consistently half those obtained with Amersham ^{125}I -PVP in matched control experiments. In all experiments the uptake was acceptably linear with time. All the experiments

discussed above were performed with the second preparation of each radiolabelled polymer (see Table 9.1.) and the results indicate a trend of decreased Endocytic Index with increased substrate size. The preliminary experiments carried out with the first batch of radiolabelled polymers supports this trend (see Fig. 10.1.) as S4 was taken up by yolk sacs (over 6.5h) approximately half as fast as Amersham ^{125}I -PVP. In this experiment the slower rate of capture of S3 relative to Amersham ^{125}I -PVP is not so obvious. The decreased rate of uptake of S3 and S4 was not due to an inhibition of the normal rate of pinosome formation, as judged by their inability to change the rate of uptake of Amersham ^{125}I -PVP (see Table 10.2.).

The preliminary experiments with S1 showed a tissue association over 6.5h that was greater than that measured for Amersham ^{125}I -PVP, and this observation was somewhat difficult to explain. Owing to poor experimental technique, the second preparation of S1 was not labelled adequately, and although it was possible to carry out experiments with this preparation the radioactivity accumulated by the yolk sac was so low that it was impossible to quantitate uptake efficiently. Uptake plots were produced, but the correlation coefficients were too low to usefully enable calculation of Endocytic Indices. It was felt that the average uptake at each time interval for all the experiments did give a useful indication of the endocytic properties of S1. These results again suggested that S1 tissue-accumulation was greater than that of Amersham ^{125}I -PVP. These results still need confirmation, but if proven what do they mean? There is now a wealth of data which supports the hypothesis that ^{125}I -PVP is a fluid-phase marker of pinocytosis in the rat visceral yolk sac and this concept is discussed at length throughout this thesis. Theoretically this means that molecular weight of PVP should not have any effect on its rate of pinocytosis unless the molecular weight becomes high enough to preclude entry of the molecule into the newly forming pinosomes. If S1 is taken up at a faster rate by

the yolk sac there would be at least three explanations of such a result:

- i small PVP molecules can bind to the plasma membrane
- ii small PVP molecules can stimulate fluid-phase pinocytosis
- iii some of the components of the S1 preparation are small enough to diffuse into the tissue by a non-pinocytic mechanism

The second possibility can be discounted as the addition of unlabelled S1 to a culture containing Amersham ^{125}I -PVP did not enhance the rate of uptake of this substrate (Table 10.2.); if anything, the Endocytic Index was marginally reduced. The third seems very unlikely as the ^{125}I -PVP molecules within the preparation are all large enough to be retained by dialysis membrane so they should not penetrate the plasma membrane. When a more active preparation of S1 is made it will be possible to investigate the binding properties of S1 and compare the extent of binding with that of Amersham ^{125}I -PVP using a culture system which includes a metabolic inhibitor or one that is performed at low temperature. It is slightly worrying that the ^{125}I -labelled S1 preparation contained substantially more [^{125}I] iodide than the other preparations (S2, S3 and S4), and there was some indication that addition of free [^{125}I] iodide to the cultures containing Amersham ^{125}I -PVP may have elevated its Endocytic Index (see Section 9.4. and Table 9.4.). Perhaps the apparent elevated tissue-accumulation of S1 is not representative of a higher rate of capture, but this speculation can only be usefully examined with another preparation of S1 that has a much higher specific activity and hopefully a lower [^{125}I] iodide content.

Before meaningful deductions can be made about the endocytic limitations of the visceral yolk sac (with regard to substrate size) this series of experiments must be expanded to include a more detailed study of the exact molecular weight distributions of the samples S1 to S4 after radiolabelling, as it is only the uptake of the ^{125}I -labelled polymers

that is measured. Although the polymer samples that we have been given greatly extend the molecular weight range of the ^{125}I -PVP that is commercially available some work is still needed to reduce the molecular weight distributions of some of the samples.

Forthcoming experiments will involve the radiolabelling and quantitation of uptake of another PVP sample that we have, S5, which has an average molecular weight of 7,000,000. The maximum size limit for an endocytosable substrate can be more easily assessed using a substrate which is spherical, as the effect of molecular weight on the configuration of a polymeric chain like PVP is quite complicated. It is hoped to investigate the effect of substrate size on endocytosis in the yolk sac using latex beads and also colloidal ^{198}Au which is commercially available in two different size ranges.

CHAPTER 11.

GENERAL DISCUSSION.

11.1. FUNCTIONAL ROLE OF PINOCYTOSIS

In the work documented in this thesis the pinocytic uptake of macromolecules and colloidal material was investigated in some depth. Although the process of pinocytosis is of enormous importance in many aspects of cell physiology, uptake of solutes may not be its only functional role. Other possible functions are as follows:-

- i) A membrane retrieval system to compensate for an increase in plasma membrane surface area caused by events such as secretory discharge.
- ii) A membrane surveillance system to facilitate the removal of defective components from the plasma membrane. This mechanism would include the internalization of hormone-receptor complexes that are no longer operational.

The internalization of extracellular material as a result of pinocytic processes whose main functional role is either of the two stated above would be merely incidental; this point is discussed by Pratten et al., (1979).

The function of pinocytosis as a membrane retrieval system has not yet been unequivocally proven, although there is considerable circumstantial evidence which supports this concept. The retrieval of discharged secretory granule membrane in the form of small pinosomes has been postulated as a result of electron microscopical observation of acinar parotid cells following stimulation of exocytosis (Herzog & Farquhar, 1977; Herzog & Miller, 1978). They were able to detect coated vesicles at the site of secretory granule fusion with the plasma membrane and these were assumed to be moving inwards. Other groups have suggested that there is reutilization of neurone membrane following neurotransmitter release by a mechanism that involves a

pinocytic uptake (Fried & Blaustein, 1978; Schaeffer & Raviola, 1978). The involvement of membrane bounded vesicles in membrane retrieval has been called into question by the stereological analysis of Cope & Williams (1973), who found that the plasma membrane of acinar parotid cells quickly returns to its original proportions following degranulation, but with no concomitant increase in the quantities of intracellular smooth membrane.

The role of a membrane shuttle system in the surveillance and removal of faulty components is entirely hypothetical but the internalization of membrane and possible exposure to lysosomal hydrolases would offer an ideal opportunity for the removal of defective components.

The rat visceral yolk sac is an excellent model for studying pinocytosis owing to its reliability, durability, and physical size. Although it is believed that pinocytosis in this tissue serves a role in embryotrophic nutrition and/or transport of immunoglobulins from mother to fetus (see Section 1.3.) there is little experimental evidence to substantiate these ideas. It is unlikely, although not impossible, that the yolk sac epithelium is a specific secretory system, so one can probably discount an involvement of pinocytosis solely in membrane retrieval. Whatever the function of pinosome formation in this tissue, its rate is extremely consistent in yolk sacs at 17.5 days of gestational age and in fact this consistency is maintained between 15.5 days and term (Ibbotson, 1978).

11.2. MECHANISMS OF PINOCYTOSIS

Should we try to separate the endocytic phenomena observed in different cell types into discrete categories? This is a question which provokes debate and one that merits a more lengthy discussion. Authors generally seem impelled to classify the pinocytic processes they are investigating as either micropinocytosis or macropinocytosis according to the classification scheme proposed by Allison & Davies (1974). The basis of this scheme is shown in Table 11.1. Perhaps the most fundamental questions that should be considered are whether or not pinocytic, or for that matter all endocytic, phenomena are actually amenable to the rigid confines of any classification.

In general terms, we know that any classification scheme which can be usefully applied to a biological situation must have sub-divisions that have relatively obvious demarcation limits. Inevitably there are phenomena which approach the boundaries between two categories and these can prove difficult to classify, but they are usually relatively rare. If the events which are difficult to classify increase in frequency, then this is the time to question the viability of the overall classification scheme. Often the scheme can be simply rectified by introducing a new category to accommodate the anomalous observations but this cannot go on ad infinitum and eventually the fundamental principles of the scheme must be questioned.

All endocytic processes that have been documented fall somewhere within a spectrum where there is a gradation in:-

- i) the size of vesicle employed to capture extracellular material
- ii) sensitivity to cytoskeletal inhibitors. (The magnitude of this effect may conceivably be directly proportional to vesicle size).

TABLE 11.1. THE CRITERIA FOR CLASSIFICATION OF ENDOCYTIC MECHANISMS ACCORDING
TO THE SCHEME OF ALLISON & DAVIES (1974)

| Event | Substrate | Vesicle/Vacuole or particle size | Requirement for Metabolic Energy | Requirement for a Cytoskeletal system | Potential for Stimulation |
|------------------|--------------------|---|-------------------------------------|--|------------------------------|
| Phagocytosis | Large Particles | 1 μ m | ++ | ++ | + |
| Macropinocytosis | Solutes | 0.3 - 30 μ m | + | + | + |
| Micropinocytosis | Solutes | Uniform diameter of approximately 70nm | - ? | - | - |

iii) sensitivity to metabolic inhibitors.

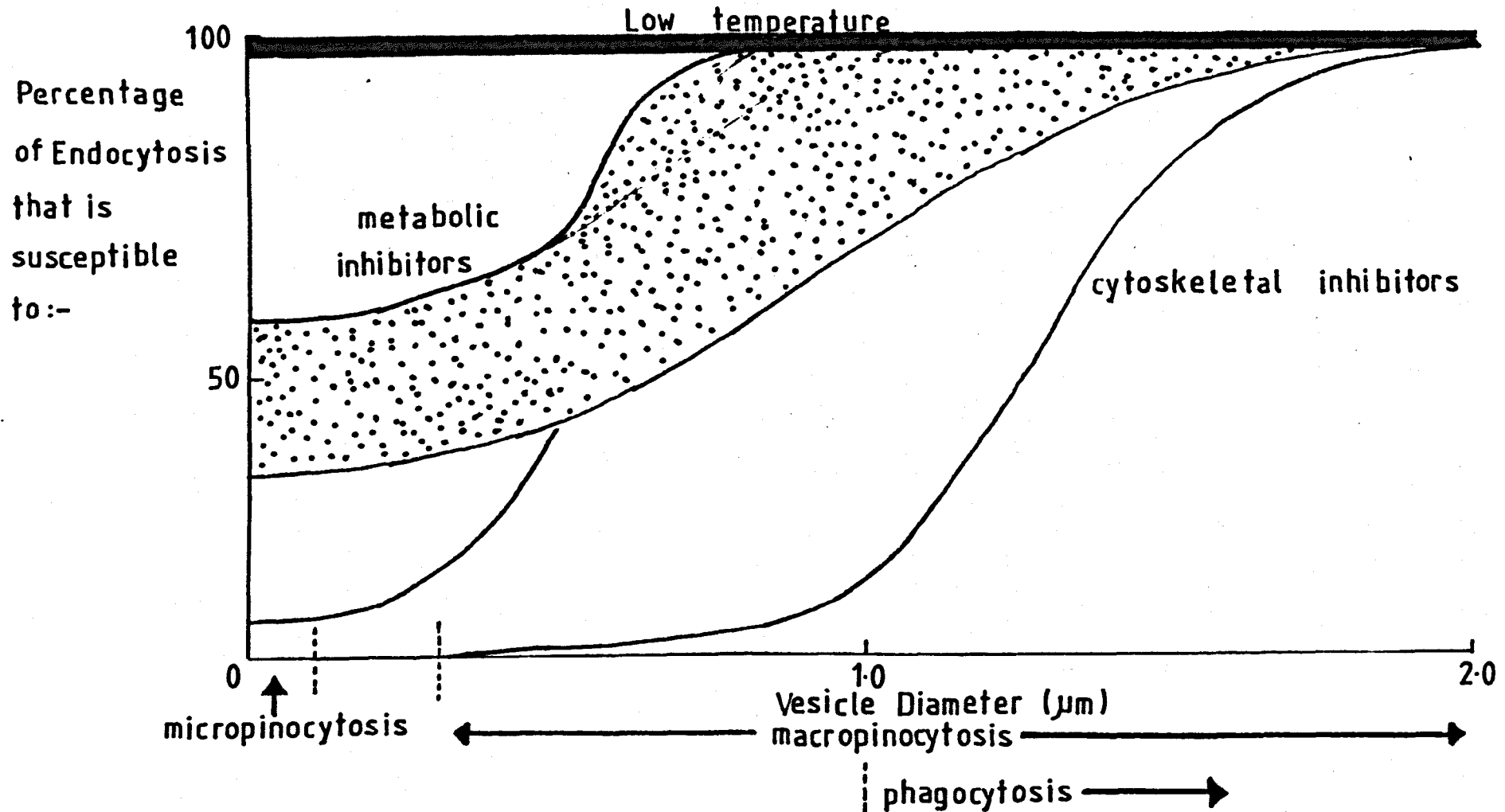
Most processes are also completely inhibited at low temperature.

In my view all the data available at the present time display continuous variation of the parameters mentioned above and the relationships between them are likely to fit within the hypothetical scheme shown in Fig.11.1, which is labelled with the "conventional" endocytic categories according to vesicle size. The relationships between metabolic and cytoskeletal dependency and vesicle size that are shown are entirely hypothetical, based simply on the following two assumptions:

1. the formation of larger vesicles requires greater cytoskeletal involvement than the formation of small vesicles which may conceivably be completely independent of this system
2. no pinocytic process is completely independent of metabolic energy, but the involvement of larger pinosomes may make the process more energy consuming.

There is direct experimental evidence which at least in part substantiates these assumptions. At present there is insufficient information available to define these relationships more comprehensively and it is hoped that the hypothetical scheme shown is sufficiently broad to contain the "true" relationships. It is felt that this diagram, with all its shortcomings, emphasizes the unity of all endocytic events, and casts some doubt on the validity of the classification scheme that has been advocated by Allison & Davies (1974). I would suggest that at present the division of pinocytosis into macropinocytosis and micropinocytosis should be discouraged, and all pinocytic processes defined more simply according to:-

FIG. 11.1. SOME THEORETICAL RELATIONSHIPS BETWEEN CERTAIN FACTORS
AND VESICLE DIAMETER



1. the vesicle size routinely observed within the tissue. (The newly formed vesicle size is the most helpful piece of information, as the extent of fusion in a system obviously plays a large part in the determination of secondary lysosome dimension).
2. the sensitivity of the system to given concentrations of metabolic and cytoskeletal inhibitors.

Although this alternative method for the description of endocytic processes does not provide a set of neat categories into which the systems under investigation can be pigeon-holed, it does remove the temptation to give an endocytic event a "label" which is not really applicable. Most processes that have been investigated do not fit exactly into the categories of macropinocytosis or micropinocytosis, but they are frequently described as such. Abandoning this terminology, with a consequent simple documentation of observations, will clarify what is becoming a very confusing situation, and facilitate unprejudiced comparison of data derived from different sources. As more information becomes available regarding the mechanisms of endocytosis operative in different cell types it will be possible to evaluate the theoretical model shown in Fig. 11.1, and then review the possibility of adoption of an acceptable classification scheme.

Historically phagocytosis and pinocytosis have always been distinguished (see Section 1.1.) and, since these processes appear to be mechanistically different this distinction seems quite justifiable. There is a reliable morphological criterion that can be used effectively to distinguish between phagocytosis and pinocytosis and this is the observation that during phagocytosis the plasma membrane spreads out to engulf a particle, whereas pinocytosis involves simple membrane invagination.

Data shown here in Chapter 4 illustrate the difficulties of trying to classify a particular endocytic process according to the scheme of Allison & Davies (1974). The dilemma that one encounters is summarized in Fig.11.2. Although it is impossible to define pinocytosis in the rat visceral yolk sac as either macropinocytosis or micropinocytosis, nevertheless it is quite acceptable to define this process using the term pinocytosis, as relatively small newly formed vesicles are involved and these are visualized as membrane invaginations.

Fig. 11.2. AN ATTEMPT TO CLASSIFY PINOCYTOSIS IN THE RAT VISCERAL YOLK SAC
ACCORDING TO THE SCHEME OF ALLISON & DAVIES (1974).

| Questions asked about uptake of solutes by the Rat Visceral yolk Sac | Answer | Conclusion | Classification |
|--|---|--|----------------|
| Vesicle Size? | Diameter 70nm | Micropinocytosis and Macropinocytosis | |
| Temperature Dependent? | Yes | Macropinocytosis | |
| Metabolic Energy Dependent? | Yes | Macropinocytosis | |
| Cytochalasin B Dependent? | Partially | Micro/Macro pinocytosis | ? |
| Colchicine Dependent? | Partially | Micro/Macro pinocytosis | |
| Stimulatable? | Yes by Mechanisms other than increased Pinosome formation | Macropinocytosis | |
| Solute Size? | 30nm diameter particles | Not Phagocytosis | |

11.3. SELECTIVITY IN PINOCYTOSIS

Capture of extracellular material is a consequence of all membrane invaginations, but the indiscriminate accumulation of substances in solution is obviously less efficient than selective uptake of material that is particularly needed by the cell.

In a pinocytic system there are two obvious levels where selection between potential substrates can occur. Firstly the size and configuration of a molecule or size of a colloidal particle must determine whether a substrate is small enough to become entrapped within pinosomes forming at the plasma membrane. Although as yet there has been no definitive study to determine the size range of substrates that can be internalized by pinocytosis, it is obvious that there will be a maximum size of substrate suitable for pinocytosis for any given cell type. Secondly, the affinity of a substrate for the plasma membrane greatly affects its rate of capture, providing it is not so large that it is excluded from the nascent pinosomes. Many studies have recently been undertaken to determine the subtleties of both membrane and substrate features that facilitate membrane binding.

The effect of molecular size of ribonuclease A on its endocytic capture has been studied in vitro (Bartholeyns & Baudhuin, 1976) and in vivo (Kooistra et al., 1977) and both studies confirmed a higher rate of capture of larger molecular weight material. Kooistra et al., 1977) used a delicate cross-linkage method to produce ribonuclease oligomers that retained their charge and biological activity and it was found that 1h after injecting monomer, dimer and oligomer into nephrectomized rats 1%, 7% and 19% respectively were recovered

per g of liver protein. All forms of ribonuclease A were preferentially accumulated within the sinusoidal cells of the liver and it was suggested that this resulted from a particular affinity for the membranes of these cells as they do not have an unusually high pinocytic rate. The positively charged lysine residues have been implicated in this interaction as acetylation reduced the rate of uptake of ribonuclease and succinylation had an even stronger effect. These studies show that the specific uptake of different molecular weight species of ribonuclease A is not a simple sieving effect, but also involves substrate-membrane interaction.

Non-specific chemical modifications of substrate may cause dramatic changes in their rate of capture. Moore and co-workers (1977) used a variety of physical and chemical treatments to modify proteins such as BSA and orosomucoid and monitored their pinocytic uptake by the rat visceral yolk sac. Non-specific modifications caused by treatment with urea, acetic acid, formaldehyde or freezing and thawing produced enhanced rates of pinocytosis and it was postulated that the changes in tertiary structure of the molecules enhanced membrane adsorption. Recently Argawal & Moore (1979) subjected BSA to specific chemical modification by dinitrophenylation, dansylation, trifluoroacetylation and N-methylation and examined its pinocytosis both in vitro (using the rat visceral yolk sac system) and in vivo. All modifications resulted in enhanced uptake of BSA, but dinitrophenylation and dansylation also decreased the degradability of BSA and this was readily apparent as these substrates accumulated within the tissue.

Certain specific pinocytic recognition systems have been studied in detail and these are summarized in Table 11.2. They are discussed extensively in three recent reviews (Goldstein & Brown 1977; Lloyd & Griffiths, 1979; Williams, 1979), so although of critical

TABLE 11.2 PINOCYTIC RECOGNITION SYSTEMS

| System | Substrate Recognition Determinant | Membrane Recognition Site | Cell Type | Reference |
|---|---|---------------------------------|---------------------------------|-------------------------------|
| <u>Galactose Determinant</u> | glycoproteins with a terminal galactosyl residue | Sialic acid terminal residue | Hepatocytes | Pricer & Ashwell (1971) |
| <u>N-acetylglucosamine/ mannose Determinant</u> | Glycoproteins with a terminal mannose, N-acetylglucosamine or glucose residue | - | Rat alveolar macrophages | Stahl <u>et al.</u> , (1978) |
| | Glycoproteins with terminal mannose and N-acetylglucosamine | - | Kupffer Cells | Achord <u>et al.</u> , (1977) |
| | Glycoproteins with terminal mannosyl or N-acetyl glucosaminy | - | Gangliosidosis skin fibroblasts | Hieber <u>et al.</u> , (1977) |
| | Bovine Ribonuclease B which has terminal mannosyl residues | - | - | Baynes & Wold (1976) |
| <u>Phosphohexose Determinant</u> | Lysosomal enzymes containing phosphomonoester | - | Human fibroblasts | Kaplan <u>et al.</u> , (1978) |
| <u>Low Density Lipoprotein Receptor</u> | Apoprotein B of LDL | Possibly a glycoprotein | fibroblasts | Goldstein & Brown (1977) |

importance to selective pinocytosis they are not discussed at length here.

Although the modification of proteins and examination of their rates of pinocytosis has helped us to understand features that are relevant to substrate/membrane interactions in many pinocytic systems there is still a great potential for the involvement of more easily definable substrate molecules which will help elucidate these mechanisms further. An obvious candidate is the PVP molecule and the effect of molecular weight on the uptake of PVP is reported in Chapter 10. Initially it was hoped to carry out experiments for inclusion in this thesis using modified PVPs of precisely defined composition. When PVPs become available which contain different proportions of substituted groups which are cationic, anionic or hydrophobic in nature, it will be possible to test their pinocytic susceptibility, using the well defined rat visceral yolk sac culture technique.

11.4. MANIPULATION OF ENDOCYTIC RATE

The rate at which a cell internalizes material via endocytosis can be influenced in two distinct ways: firstly by changing the affinity of the ingested material for the plasma membrane, and secondly by an alteration in the actual rate of endosome formation.

11.4.1. Carrier-mediated Endocytosis

Adsorption of material to the plasma membrane greatly enhances its rate of endocytosis, and in the previous Section (11.3.) many of the known physiological recognition systems are described. Sometimes it is possible to increase the endocytic uptake of a particular substance (one that is not normally selectively captured) by some artificial means. Over the last few years many reports have appeared, directly or indirectly relating to the suitability of liposomes as drug carriers (reviewed by Tyrell *et al.*, 1976; Gregoriadis & Allison, 1979). These synthetic lipid membranes were originally used as model systems to investigate membrane phenomena, but their usefulness for the entrapment of material, either in the aqueous phase or in association with the lipid itself, soon became obvious. Depending on their composition liposomes either fuse with the plasma membrane and discharge their contents into the cytosol, or are engulfed by endocytosis and subsequently degraded in the lysosomes. Recently Gregoriadis (1978) has discussed the potential of liposomes as enzyme carriers having the potential to alleviate the specific problems of lysosomal diseases (outlined in Section 1.1.2).

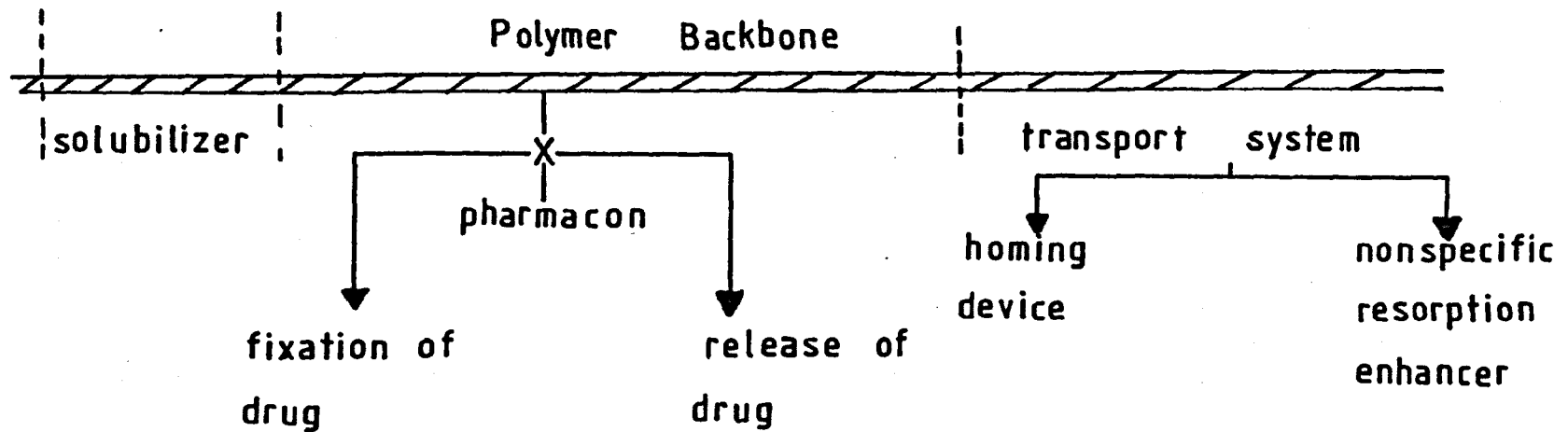
Synthetic polymers offer another possible vehicle for the transport of certain drugs and it is only very recently that they have been considered as a means to solve therapeutic problems. Both of these approaches are still in their infancy and there are many problems to

be overcome, but both yield new and exciting possibilities for making drug administration more specific.

The efficient use of a carrier system such as liposomes or polymers requires a detailed knowledge of its relationship to the endocytic mechanism, and the majority of the first wave of investigations with liposomes were carried out in vivo with no consideration of events at the cellular level. More recently attempts have been made to define the mechanisms of liposomal incorporation into cells (Poste & Papahadjopolous, 1979; Batzri & Korn 1975; Bridges et al., 1978; Hoekstra et al., 1978; Bayer et al., 1979), but the interpretation of the data obtained can often be difficult, owing sometimes to poor experimental design. Information on features such as metabolic temperature, and cytoskeletal dependency, (as carried out here for the rat visceral yolk sac, Chapter 4,), is invaluable before one can begin to explore the involvement of carrier systems in these processes.

Some of the experiments reported in this thesis are directly relevant to attempts to use synthetic polymers as a drug transport mechanism. Two of the compounds utilized here, polylysine and DIVEMA, have actually been used as carriers to increase the rate of incorporation of methotrexate. Other substances may be potential carriers, the inert PVP molecule being one obvious example and, if they are ever used as such, the more information that is available regarding the endocytic properties of the carrier molecules themselves the easier it will be to predict the destiny of the carrier-complex. Fig. 11.3. shows a model for such a carrier system. Devising the so-called transport system of the molecule depends entirely on basic understanding of endocytic recognition systems. From general experience one might expect the non-specific enhancers to involve

FIG. 11.3. A MODEL FOR PHARMACOLOGICALLY ACTIVE POLYMERS



(After Ringsdorf, 1975)

cationic groups and/or hydrophobic groups and the most specific homing devices to involve the integration of some cell-specific antibody into the molecule. Comparative studies of endocytosis will help to elucidate other cell-specific membrane features that might be exploited for specific targeting. It might be possible to single out neoplastic cells in this way as they are known to have certain unique membrane properties. Looking to the future it seems likely that the in vitro endocytic system will provide a useful method for studying pharmacon-polymer behaviour.

11.4.2. Control of Basal Endocytic Rate

The rate at which cells engulf material by phagocytosis is probably determined by the extracellular concentration of suitable particles and also the finite capacity of the cells to take them up and degrade them. Phagocytosis is thought to be initiated by particle attachment to the membrane, and subsequent internalization may be mediated by one or more secondary messengers (see Section 1.1.1.). In contrast pinocytic uptake in many cell types appears to continue at constant rate for long periods (when measured in simple in vitro systems). This can be thought of as the basal or intrinsic rate of pinocytosis for any cell line. Are there a set of conditions which modulate this basal rate to give elevated or depressed rates of internalization according to the changing requirements of the cell? The answer to this question is far from clear and whether or not the incessant vesicle formation that can be visualized cinematographically is a precisely controlled chain of events is not yet understood.

The word pinocytosis describes the uptake of small droplets

of extracellular fluid, but from the foregoing Sections it has become apparent that the interaction of the cell with the substances contained within this fluid is of fundamental importance in determining the differential rates of accumulation of extracellular materials. As binding retains its importance in pinocytosis, perhaps this is still the key to rate control, at least for the uptake of specific substances. It has been suggested that pinosomes may form which contain a negligible amount of extracellular fluid and it is envisaged that such vesicles are involved in the uptake of specific macromolecules such as immunoglobulins. It seems likely (although not essential) that such a system would be initiated by membrane binding. Although modulation of pinocytic rate may be mediated by a direct extracellular trigger in the form of the substrate itself, an alternative may well include an ultimate rate-determining mechanism which is intracellular. If pinocytic activity were, for example, under some hormonal control, interaction of hormone with the plasma membrane might then produce physiological changes within the cell that could then feed back and influence the rate of pinosome formation at the plasma membrane. By analogy with the secretory mechanism it is not unreasonable to suppose that intracellular concentrations of appropriate compounds may be of paramount importance in the fine control of the rate of pinosome formation.

Between the ages of 15.5 and 20.5 days of gestation the rat visceral yolk sac displays a constant basal rate of fluid-phase pinocytosis, which is approximately equal to a clearance of $2\mu\text{l}/\text{mg protein}/\text{h}$. It is not known if this process is regulated in any way under normal physiological conditions, but it was disappointing to find that addition or removal of potential modifiers (see Chapter 4) only reduced the rate of fluid uptake. Conclusion that any of the compounds

investigated were influencing pinosome formation in a physiological way is impossible. The failure to find a stimulator of pinocytosis in this system may relate to the suitability of the model for studying pinocytic rate control.

There are relatively few known stimulators of fluid-phase pinocytosis in any system. Concanavalin A appears to stimulate the uptake of horseradish peroxidase and ¹²⁵I-BSA by mouse peritoneal macrophages (Edelson & Cohn, 1974 a), but this effect is combined with concanavalin A inhibition of pinosome-lysosome fusion (Edelson & Cohn, 1974 b), the stimulation of lysosomal enzyme synthesis (Goldman & Raz, 1975) and the inhibition of the phagocytosis of latex particles by mouse peritoneal macrophages (Friend et al., 1975). Another pinocytosis induction process has been mentioned earlier and this is the initiation of channel formation in Amoeba proteus by inorganic salts such as sodium chloride (Chapman-Andresen, 1965). This situation is slightly different from those mentioned above as the amoeba has no basal level of ongoing pinocytosis, and once induced the pinocytic cycle only continues for 20-30min. Very recently Davies & Ross (1978) reported that factors derived from whole blood serum and from platelets stimulate the formation of pinosomes in monkey aortic smooth muscle cells.

The factors that govern pinocytic rate are still a mystery, although some rate control is probably operational. Cells in culture at different degrees of confluence exhibit different rates of pinocytosis (Prinz et al., 1978) and certain cell types appear to be more active than others (Pratten et al., 1979), so there is some variability in pinocytic rate albeit finely controlled. If, as suggested by Pratten et al., 1979), there is some degree of uniformity in the rate of

pinosome formation in many different cell types (when uptake is expressed in terms of $\mu\text{l}/\text{mg protein/h}$) it may be inferred that for at least some cell types a finely tuned rate control mechanism is not operational from day to day.

11.5. MEMBRANE CONSIDERATIONS

In any study of pinocytosis it is essential to consider membrane involvement in the overall scheme of events. The region of the plasma membrane destined to form the pinosome predetermines the nature of substances (and quantity of such substances) that will enter by an adsorptive mechanism. Once the vesicle is discharged into the cytoplasm its membrane determines the fate of the vesicle in terms of fusion with other organelles or re-fusion with the plasma membrane. A large proportion of a cell's surface area can be internalized in a short time if it is actively pinocytosing so that membrane recycling back to, or rapid replacement of, the plasma membrane is vital if pinocytosis is to be maintained for long periods.

11.5.1. Membrane Composition

It is interesting to consider whether pinosome formation involves the random internalization of undefined regions of plasma membrane or alternatively entails a more specific internalization of preselected areas. The fact that phagocytosis did not change the rate of transport of adenosine or adenine in rabbit polymorphonuclear leucocytes or adenine or lysine in alveolar macrophages led Tsan & Berlin (1971) to the conclusion that the membrane was a mosaic in composition with preselection against the proteins involved in the active transport system during phagocytic invagination. Other reports tend to conflict with this suggestion. Dunham *et al.*, (1974) investigated the relationship between phagocytic activity of polymorphonuclear phagocytes and potassium and amino acid transport. After ingesting latex or zymosan or surface binding of zymosan in cytochalasin B inhibited cells, the potassium pump was inhibited 50.75% and the uptake of lysine was also retarded. These results were interpreted as

indicating that the membrane structure is homogeneous and that phagocytosis results in the ingestion of the transport sites. As surface binding of zymosan was sufficient to inhibit solute transport it was suggested that the surface attachment stage of particle uptake was a key stage in the loss of normal membrane function. De Bruyn et al., (1978) mapped sialic acid sites on the luminal surface of endothelial cells using polycationic ferritin, and found that sialo-containing components were evenly distributed within the membrane except at the site of endocytic vesicle formation. Selection for the internalization of specific membrane components has been observed in the case of concanavalin A uptake. Here the initial binding of concanavalin A appears to be a random process, but it is followed by post-binding migration of the receptor-substrate complex into groups or clumps (Yahara & Edelman, 1973; Williams et al., 1977) Perhaps the localization of certain membrane components in a small area of the plasma-membrane causes the membrane distribution necessary for the initial stages of invagination.

Relatively few studies have been undertaken to determine the membrane compositions of the different vesicle types that are integral components of an endocytic system (pinosomes, secondary lysosomes, primary lysosomes etc.) Bode et al., (1976) isolated various membranes from rat kidney and looked at the composition of pinocytic vesicles, lysosomes, the microvillous brush border and the baso-lateral plasma-membrane. The pinocytic membranes had a higher concentration of acid phospholipids and glycoproteins than the brush border microvilli and it was suggested that these may be of importance as non-specific binding sites during pinocytosis.

The differences observed in protein, glycoprotein and lipid composition of the external and internal membranes were surprisingly large. With the known origin of pinosomes, the membrane composition should be at least partially compatible with that of the plasma membrane and this was not so in this case. To explain their observations the authors proposed independent synthesis of pinosome membrane; an idea that is not without many difficulties. The current ideas relating to membrane flow and interconversions among endomembranes have recently been reviewed (Morre et al., 1979).

As yet no experiments have been undertaken to try and define membrane compositions in the rat visceral yolk sac. Using techniques of cell fractionation and vesiculation of the plasma membrane such a study is now feasible and would be very worthwhile.

11.5.2. Vesicle Fusion

The phenomenon of membrane fusion is of enormous importance to many aspects of physiology, both at the cellular and subcellular levels. Poste & Allison (1973) discuss the process in general terms. Membrane fusion is an integral feature of endocytosis. The first fusion event occurs at the plasma membrane, when the lips of the forming vesicle meet. This is followed by a series of controlled intracellular intervesicle fusions, where the incoming vesicle may fuse with its siblings, with secondary lysosomes or primary lysosomes. Fusion with other intracellular organelles is in some way prevented, although reincorporation into the plasma membrane may be possible. In some cases the endocytic process may be involved in transport of material across the cell and here

it is possible that the interaction of pinosome with lysosome may be prevented to counteract the possibility of substrate degradation.

Factors that encourage, permit or prevent two membranes fusing are still not entirely clear, although the process would seem to occur by unmasking and subsequent intermixing of phospholipids of adjacent membranes. Studies with Tetrahymena have indicated that there is a grouping of intramembrane particles into a defined "rosette" pattern at the site of secretory granule fusion with the plasma-membrane and this appears to be a recognition system for the initial fusion event (Satir, 1974). These designated fusion sites have not been identified in other cell types. Many freeze-fracture studies of secretory cells actively engaged in secretion show distinct changes in the membrane appearance. As the secretory granule approaches and pushes into the plasma-membrane, a dome appears which is free of intramembraneous particles that represent membrane-spanning amphipathic proteins. These particles are evenly distributed throughout the rest of the membrane and form a ring at the boundary of the dome. It has been postulated that migration of intramembraneous particles out of the area of contact exposes the phospholipids which may then be attacked by phospholipases with the result that the stability of the membranes is diminished and fusion occurs. A recent freeze-fracture study of a murine plasmacytoma line engaged in the endocytosis of concanavalin A showed similar particle-free areas which were surrounded by intramembraneous particle necklaces (Hatae & Bendetti, 1978), so this phenomenon is not restricted to the secretory event.

Although artificial model systems have been used to study fusion events, the endocytic system provides an ideal opportunity

to study a natural fusion sequence in a much more physiological environment. The fusion of incoming pinosomes with lysosomes can be monitored very easily using degradable markers, and the potential of possible disruptive agents examined.

Polyglutamic acid, one of the substances used in this thesis as a potential modifier of pinosome formation, has already been shown to inhibit vesicle fusion in cells when used at the much higher concentration of 200 $\mu\text{g/ml}$ (Hart & Young, 1978; 1979). Similarly, the lectin concanavalin A can inhibit vesicle fusion in mouse peritoneal macrophages (Edelson & Cohn, 1974 b).

11.5.3. Membrane Recycling

For many years it has been obvious to everyone working in the field of endocytosis that many cells internalize large quantities of their surface membrane in the form of endocytic vacuoles and such internalization is often sustained for many hours or even days. Since the cells involved in endocytosis maintain their integrity, a dynamic situation must exist whereby recycling of membrane components compensates for the uptake processes. The problem was highlighted a few years ago by Steinman et al., (1976) who undertook a detailed stereological study of membrane flow in mouse peritoneal macrophages and found that macrophages ingesting horseradish peroxidase in vitro engulf 190% of their surface area and 26% of their volume per hour. At this time there was no experimental evidence to suggest how such rapid membrane replenishment could occur in an endocytic system although similar problems (excess membrane retrieval from the cell surface) were being investigated in exocytic (secretory) systems. It has been shown that membrane generation by rapid synthesis of new components is not possible in such a short space of time, even if such a mechanism

would be energetically economical. A colleague and I (Duncan & Pratten, 1977) suggested a theoretical model whereby the membrane distributions could be adequately conserved by the recycling of internalized plasma-membrane in some form of intact unit. The process of vesicle fusion was thought to provide the ideal situation for such a mechanism to operate. When two spherical vesicles fuse, the simple geometry of the situation means that in order for the resultant vesicle to be spherical there must either be an increase in the incoming volume or a decrease in the incoming surface area. At the instant of fusion both the incoming surface area and volume are conserved and this results in the formation of a non-turgid vesicle with a ruffled membrane. Influx of fluid into this vesicle would only add to the overall problem of fluid removal, so membrane loss, say by a process of budding, either inwards into the vacuole or outwards into the cytoplasm may be the mechanism of regaining spherical shape. The transient appearance of a "ruffled" vacuole would provide the ideal configuration for membrane loss by budding with minimal energy requirements.

The concept of such a process was not particularly revolutionary, but what was particularly interesting were the actual quantities of membrane that could theoretically be liberated by such an event. When two vesicles of equal diameter fuse approximately 20% of the incoming surface area will be released if the sphericity of the new vesicle is resumed with no increase in volume. One fusion event could therefore release 1/5th the incoming surface area for possible return to the cell surface and utilization in the construction of newly forming pinosomes. However, vesicle fusion is not an isolated

event, in fact a multitude of fusions must occur to form the larger vacuoles visualized within pinocytic cells if the vesicles forming at the membrane are relatively small (70-100 nm in diameter). The magnitude of the potential output of membrane from such an extensive fusion sequence is well illustrated by the following example. If 128 vesicles of radius 35nm fuse progressively, one vacuole of radius 175nm can be formed with equivalent volume and 80.5% of the internalized surface area liberated for reutilization.

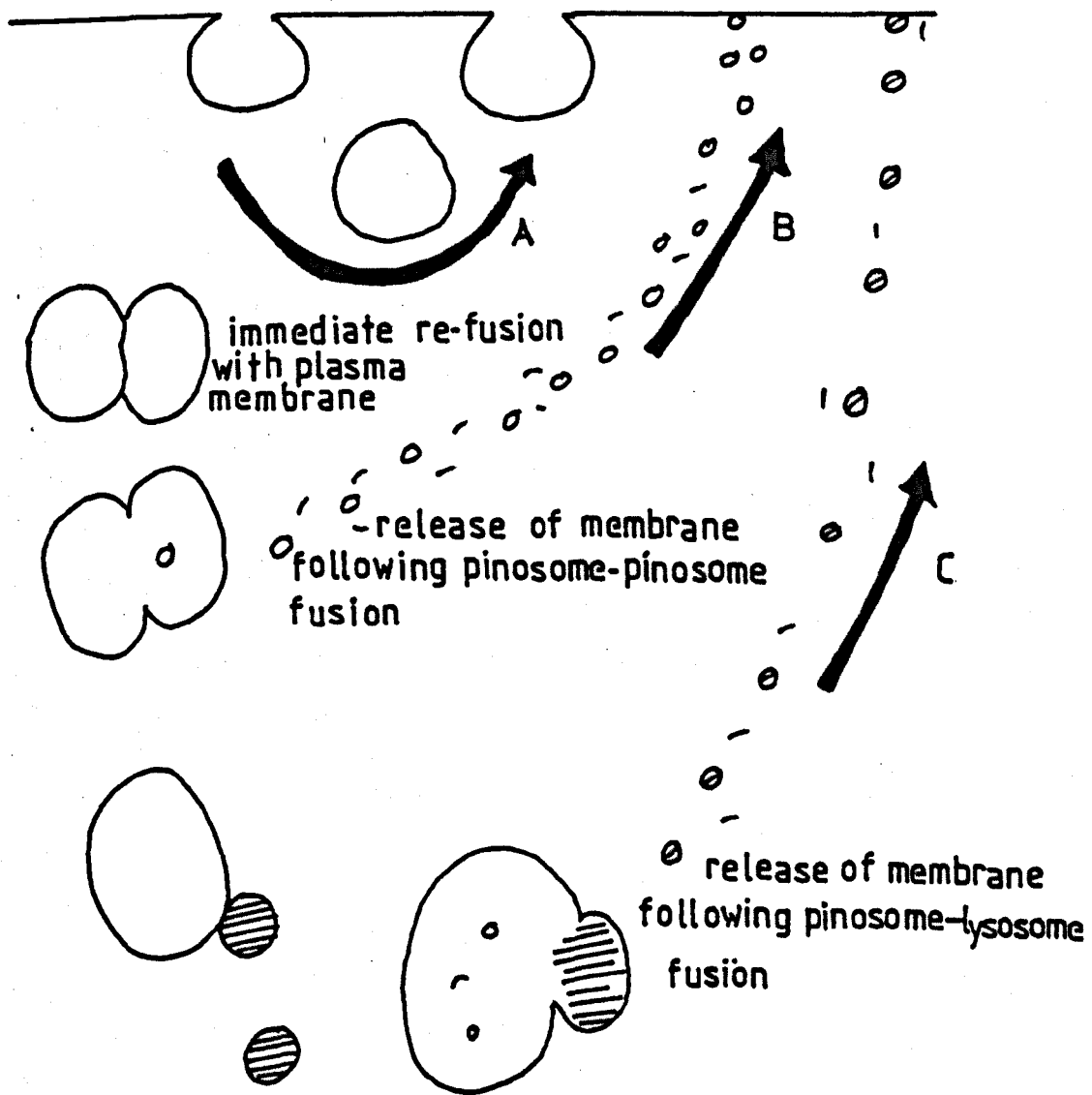
This concept of membrane recycling becomes less appealing if substantial quantities of substrate are also lost from the incoming vesicles. Perhaps the size of the returning membrane units may be sufficiently small, or if vesicular, have little volume relative to their surface area so the loss of solutes present in the bulk liquid phase would be minimized. Additionally, if the membrane composition was preselected in some way the loss of membrane-bound substrate could be totally avoided. The recycling of membrane following entry into the lysosomal system has the added complication that lysosomal enzymes may also be lost, and in fact it has been suggested that an increased loss of specific lysosomal enzymes via this route may be a possible cause of the condition called I-cell disease in which several lysosomal enzymes are known to be deficient (Lloyd, 1977 b). If the majority of membrane recycling occurred before this time the problem would be largely overcome, but it seems likely that some membrane recycling would be inevitable both before and after entry into the lysosomal compartment.

Tulkens et al., (1977) investigated the fate of endocytosed plasma membrane in rat fibroblasts using an ingenious experimental

technique which has confirmed the existence of a membrane recycling system in these cells. Rat fibroblasts were incubated with fluorescein-labelled IgG, obtained from control animals, and this was internalized by adsorptive endocytosis and accumulated in the lysosomes. In contrast an anti-plasma membrane IgG (labelled with [^3H] acetic anhydride) was accumulated by the cells, but fractionation experiments showed it to be exclusively associated with the plasma membrane marker 5'-nucleotidase. These results indicated that either the anti-plasma membrane IgG reacted with components of the membrane that were not internalized or that the whole membrane was involved in pinosome formation but that membrane carrying anti-plasma membrane IgG was recycled rapidly back to the cell surface. Cells were then incubated with fluorescein labelled goat (anti-rabbit) IgG (which was also endocytosed into the lysosomal compartment) and subsequently washed and reincubated with rabbit-anti-plasma membrane IgG for periods of 24h. Substantial amounts of goat (anti-rabbit) IgG of higher molecular weight appeared in the culture fluid during this time suggesting the release of antigen-antibody complexes. The data indicate that the rabbit anti-plasma-membrane IgG was internalized and transported to the lysosomes, where, when present in excess it reacted with the goat (anti-rabbit) IgG and carried it rapidly back to the membrane surface. The continuous interchange of membrane between the cell surface and lysosomal compartment is envisaged as a shuttle system. Fig.11.4. shows a model which describes the pathways for membrane flow that probably operate in most endocytic cells. If substantial quantities of substrate are rapidly returned to the extracellular fluid via routes A or B the tissue levels of substrate accumulated over a given period of time will obviously be an underestimate

of the pinocytic rate of the system. However, the ability of a cell or tissue to concentrate substrate within itself can be justifiably used as a measure of its effective pinocytic capacity for that particular substrate.

FIG. 11.4. THEORETICAL ROUTES OF MEMBRANE RECYCLING



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APPENDIX I

Computer Programme to Calculate the Uptake of Non-Digestible Substrates

```
5 REM KEW 125I-PVP PROGRAM,MODIFIED NOV.,1974
10 DIM H(15),I(15),L(15),R(15)
12 IF W=1 THEN 84
13 LET W=1
16 PRINT "NON-DIGESTIBLE PROG. ENTERED"
17 PRINT "EXPT.NO. (DIGITS ONLY) = ";
18 INPUT Z
19 PRINT " BACKGROUND IN CPM = ";
20 INPUT A
35 PRINT " COUNTING TIME FOR EACH ML OF MEDIUM, SECS = " ;
40 INPUT C
45 PRINT "COUNTING TIME FOR EACH ML OF YS SOLUTION, SECS = " ;
50 INPUT D
75 PRINT " NO. OF POINTS IN PLOT = " ;
80 INPUT G
82 STOP
84 LET W =,0
95 FOR X = 1 TO G
98 INPUT H(X),I(X),K(X),L(X)
99 NEXT X
100 FOR X =1 TO G
101 LET M = (I(X)*60/C) -A
135 LET Q = ( (K(X)*60/D) - A)*5
136 LET N = M + Q/20
140 LET R(X) = (Q*1000)/N*L(X) )
145 PRINT FRE (5) ; H(X), FRE(6);R(X)
150 NEXT X
153 PRINT FRE(5) ; -1, FRE(6) ; Z
155 DRSPEC TO OWN
157 PRINT " "
158 PRINT " "
160 PRINT " INCUBATION TIME (HOURS) ",
      " PROTEIN IN YS "," UPTAKE "
165 PRINT
```

c ont....

```
190 FOR X = 1 TO G
195 PRINT "    ", FRE(5);H(X), FRE(6);L(X), FRE(6);R(X)
200 NEXT X
205 STOP
```

Input Data

H(X) Duration of yolk sac incubation period (h)
I(X) Mean radioactivity count per 30s for 1.0ml of incubation medium
K(X) Mean radioactivity count per 5min for 1.0ml of yolk sac solution
L(X) Total protein content of the yolk sac (mg)

APPENDIX II

Computer Programme to Calculate the Uptake of Digestible Substrates

```
1 REM PROTEIN PROGRAM, REVISED OUTPUT, NOV. 1974
10 DIM E(12),H(12),I(12),J(12),K(12),L(12),S(12)
12 IF W = 1 THEN 84
13 LET W = 1
14 PRINT " PROTEIN PROGRAM ENTERED "
15 PRINT " EXPT. NO. (DIGITS ONLY) = " ;
16 INPUT V
19 PRINT " BACKGROUND IN CPM = " ;
20 INPUT A
25 PRINT " PERCENTAGE SOLUBLES IN PREP. = " ;
30 INPUT B
35 PRINT " COUNTING TIME MEDIUM TOTALS,SECS = " ;
40 INPUT C
41 PRINT " COUNTING TIME MEDIUM SOLUBLES,SECS = ";
42 INPUT Z
45 PRINT " YS COUNTING TIME,SECS = " ;
50 INPUT D
60 PRINT " CORRECTION FACTOR FOR MEDIUM TOTALS = " ;
65 INPUT Y
67 PRINT " CORRECTION FACTOR FOR MEDIUM SOLUBLES = " ;
68 INPUT R
75 PRINT " NO. OF POINTS IN PLOT = " ;
80 INPUT G
82 STOP
84 LET W = 0
95 FOR X = 1 TO G
98 INPUT H(X),I(X),J(X),K(X),L(X),
99 NEXT X
100 FOR X = 1 TO G
101 LET M = (I(X)*60/C - A)*Y
102 LET N = (J(X)*60/Z - A)*R
105 LET O = N - (M*B/100)
135 LET Q = (K(X)*60/D - A)*5
136 LET P = (M - N) + O/2
137 LET F = ( (10*O) + Q)*1000
```

cont.....

```
138 LET E(X) = (Q*1000 ) / (L(X)*P)
139 LET S(X) = F/(L(X)*P)
140 PRINT FRE (5) ; H(X), FRE(6) ; S(X)
150 NEXT X
151 PRINT FRE (5) ; -1,FRE ( ) ; V
152 DRESPEC TO OWN
153 PRINT      "  "
154 PRINT      "  "
155 PRINT " INCUBATION ", " PROTEIN ",
      " MICROLITRES ", " UPTAKE "
160 PRINT " TIME (HOURS ) ", " IN YS ", " PER MG YS "
190 FOR X = 1 TO G
195 PRINT FRE(5) ; H(X), FRE (%) ;
196 PRINT L(X), FRE(6) ; E(X), FRE(6)
200 NEXT X
205 STOP
```

Input Data

H(X) Duration of yolk sac incubation period (h)
I(X) Mean radioactivity count for 1.0ml of incubation medium
J(X) Mean TCA-soluble radioactivity count for 1.0ml of incubation
medium
K(X) Mean radioactivity count for 1.0ml of yolk sac solution
L(X) Total protein content of the yolk sac (mg)